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1	Your reference	MRH/P15700 19 DEC 1998
2	Patent application number	9827921.9
3	Full name, address and postcode of the applicant	**ML Laboratories Plc** **60 London Road** **St Albans** **AL1 1NG**
	Patents ADP number	7152800004
	State of incorporation	**UK**
4	Title of the invention	**IMMUNOSUPPRESSION**
5	Name of agent	**HARRISON GODDARD FOOTE**
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	Patents ADP number	**14571001**

6	Priority applications	Country	Priority App No	Date of Filing

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7	Parent application (eg Divisional)	Earlier Application No	Date of Filing
8	Statement of Inventorship Needed?		
9	Number of sheets for any of the following (not counting copies of same document)		
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	Description	**28**	
	Claims	-	
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12	Name and daytime telephone number of person to contact in the United Kingdom	**Michael R Harrison** **0113 225 8350**	

IMMUNOSUPPRESSION

1. FIELD OF THE INVENTION

This invention relates to immunosuppression and, more particularly, to immunosuppression in the context of xenotransplantation.

2. BACKGROUND TO THE INVENTION

Despite the established success of allogeneic organ transplantation, the increasing disparity between the supply and demand of organs must be overcome. Increasing the supply of allogeneic organs does not offer a satisfactory solution because even if all usable organs were transplanted this would still not meet the existing demand (1,2). This has led to a resurgence of interest in xenotransplantation (the transplantation of organs between animals of different species) as a viable and attractive alternative.

Xenotransplantation research has recently focused on the pig as a suitable animal donor in terms of size, physiological compatibility and breeding characteristics (3,4). Until recently however , discordant xenotransplantation has been limited by the inevitable occurrence of humorally-mediated hyperacute rejection (HAR) which rapidly triggers organ rejection upon revascularisation. HAR is the fate of most organs transplanted between discordant species. Recently, significant advances have been made in understanding the immunological basis of HAR, and many approaches have been employed to overcome it. Of significance, a variety of transgenic strategies are currently being employed including the expression of regulators of complement activity on porcine endothelial cells (5). It is foreseeable that short-term xenograft survival will soon be achieved (6). The recent advances in overcoming HAR have highlighted subsequent immunological barriers which must be surmounted to enable long-term xenograft survival. Both humoral and cellular arms of the immune response appear to play a role in the downstream events of immunological rejection. Clearly the most important of which is the existence of a formidable T cell mediated rejection response (7-11) previously

obscured by the dominant role of HAR. *In vitro* , human T cells have been demonstrated to play a central role in the recognition of xenogeneic cells (7,8,12) following sensitisation via the direct and indirect T cell activation pathways, which have been well documented for allorecognition and allograft rejection (13). Knowledge of the cellular mechanisms underlying allorejection has provided an important basis for the investigation of the T cell mediated xenoresponse.

At present, the major therapies to prevent cell mediated rejection of organ transplants rely on systemic immunonosuppressive drugs or monoclonal antibody (Mab) therapy directed against targets such as CD3, CD4, CD25, (14). Following reports that strong T cell xenoresponses can be generated *in vitro* (7,8,12), control of xenograft rejection may require levels of immunosuppresion much greater than the current standard doses. Such a strategy would not be desired in a xenograft context. Drugs must be taken for life, depress the entire immune system and result in an increased risk of infection and susceptibility to cancer (14). For the applicability of xenotransplantation to the clinic, targeting graft-specific strategies for tolerance induction/immunosuppression would clearly be highly advantageous. Whilst this has been difficult to achieve in an allotransplant context, xenotransplantation offers greater potential - with differences between species providing the option for the generation of reagents that are truly graft specific. In addition, there is the opportunity for the manipulation of both the porcine donor organ, and the human recipient's immune system, prior to transplantation (1).

3. DETAILED BACKGROUND

3.1 T cell activation and proliferation

Optimal proliferation of T cells, although initiated via ligation of the antigen specific CD3/TCR complex (Signal 1) requires additional costimulatory signals (Signal 2) (15,16,17) which are usually supplied by the antigen presenting cell (APC). Whilst antigenic stimulation of T cells in the presence of signal 2 induces T cell activation and proliferation (18), exposure of T cells to MHC-antigen complexes in their absence leads to aborted T cell proliferation and the development of clonal anergy (19,20).

Manipulation of APC by aldehyde fixation (20,21) or heat treatment (19) has been demonstrated to abrogate the ability of such cells to activate alloreactive T cells, without altering levels of MHC-II surface expression. Thus T cell receptor occupancy alone is insufficient to fully activate the T cell (17). Anergic T cells are best characterised by their lack of IL-2 production and their continued inability to produce IL-2 on subsequent exposure to antigen (22). Thus, confirming the two signal model of activation as predicted by Lafferty *et al* (23). For T cells to respond to a given antigenic stimulus, multiple activation signals are required from the APC (23).

The *in vivo* induction of T cell anergy in the absence of a secondary signal was first demonstrated by Jenkins and Schwartz in 1986 (24) using chemically fixed APC to present specific peptide to CD4 T helper clones. A multitude of *in vitro* and *in vivo* data has since been produced supporting the hypothesis that signal 1 in isolation fails to activate T cells (22), and that costimulatory signalling results from contact with other cells rather than via soluble factors. Fibroblasts transfected with human Class II MHC molecules, but not expressing the appropriate CS signals (lacking signal 2) can efficiently present antigen to class II restricted CD4 T cell clones, but these fail to cause antigen specific T cell proliferation, rendering cells anergic. The context in which T cells first encounter antigen therefore has an important bearing on subsequent immune responsiveness.

Thus, costimulatory molecules are essential for T cell activation and multiplication and result from interactions between receptors on T cells and their ligands expressed on the APC. The costimulatory signal itself, however, is neither antigen specific nor MHC restricted (25). In recent years the molecular interactions involved in mediating costimulation have been well defined. The two key pathways involve (i) B7-1, B7-2 (members of the B7 family) and (ii) CD40, which are expressed on the APC, and their counter-receptors CD28 and CD40 ligand (CD40L) respectively expressed on T cells. A large body of evidence, both *in vivo* and *in vitro* , clearly defines the crucial roles played by B7-1, B7-2 and CD40 in providing T cell costimulation (26-36). Furthermore, the

simultaneous blockade of signalling via CD28-B7 and CD40-CD40L in an allotransplant context prevented the onset of allograft rejection (37,38). *In vivo* , targeting the B7/CD28 interaction has been shown to prevent T cell sensitisation to graft antigen, thereby prolonging graft survival (38,39).

T cells can be sensitised against xenoantigens via one of two pathways - the direct and indirect pathways, which are analogous to the well documented T cell activation pathways against alloantigens (Figure 1). Direct recognition requires that the recipient T cells recognise intact xeno MHC-molecules complexed with peptide on donor stimulator cells. In contrast, indirect recognition requires that recipient APC process the xenoantigen prior to presentation to recipient T cells in the context of recipient MHC II. Self MHC II restricted T cells with specificity for the xenoantigen will recognise the peptide and respond. Whilst the majority of data reported is of indirect xenorecognition responses, cell mediated rejection via the direct route has also been documented (7,8,9,11,12,40,41,42). Vigorous human T cell proliferative responses directed against porcine tissues *in vitro* have been documented from studies both in this laboratory and others.

3.2 Costimulatory molecules

The crucial role played by costimulatory molecules in determining the result of TCR-CD3 receptor engagement with MHC and peptides has been demonstrated extensively both *in vivo* and *in vitro* . Anti-costimulatory molecule strategies aimed at either the receptors or their ligands are being used as therapeutic strategies for altering the immune response. Such approaches have been tested in model transplant systems to alter cell mediated responses thereby preventing graft rejection (14,37,38,43-47).

B7-1 (B7/BB1, CD80) and B7-2 (CD86) both belong to the Immunoglobulin superfamily and are heavily glycosylated transmembrane proteins (25). B7-1, a B cell activation molecule was first identified in 1981 (27), followed by B7-2 in 1993 (49). Both human B7-1 and B7-2, and the murine homologues have now been cloned and functionally

characterised (25) . B7-1 and B7-2 are constitutively expressed on splenic and blood dendritic cells and are induced on B cells and monocytes upon activation (34,50,). B7-1 and 2 are highly homologous and are the natural ligands for the T cell antigen CD28 (50). Cytotoxic T lymphocyte antigen-4 (CTLA-4), a cell surface glycoprotein has been identified as a second receptor for the B7 family of molecules (51) and is homologous to CD28 with 31% sequence identity. Both B7 isoforms bind to CTLA-4 with higher affinity than to CD28 (30,50,52). Whilst CD28-B7 receptor engagement results in an APC-derived costimulatory signal involved in antigen specific IL-2 production both *in vivo* and *in vitro* (53,54), CTLA4 appears to function as a negative regulator of T cell activation (55, 56, 57). Cross-linking by anti-CTLA4 antibodies has been demonstrated to antagonise CD28 ligation (58) and, in addition, CTLA4 knock-out mice die due to uncontrolled lymphocyte proliferation within the first few weeks of life (59). Thus, CTLA4 ligation is thought to be crucial for the maintenance and regulation of immune responses. The underlying mechanisms have not, however, been clearly defined.

Among costimulatory molecules, the B7 family appears to be unique, since ligation by CD28 of either B7-1 or B7-2 is both necessary and sufficient to prevent the induction of anergy (34). The CD28-B7 interaction is thought to deliver crucial signals to sustain proliferation of activated T cells. These observations are supported by *in vitro* data showing that whilst cells deficient in B7 fail to stimulate a primary MLR, transfectants expressing high levels of B7 gained the capacity to stimulate the production of IL-2 by alloreactve T cells and to co-stimulate a polyclonal population of purified T cells cultured with immobilised anti-CD3 Mab (31). Artificial APC generated by stably transfecting NIH-3T3 cells with HLA-DR7, B7 or both, clearly demonstrated that following presentation of tetanus toxoid (TT) optimal T cell proliferation and IL-2 production resulted only when both molecules were present. In the absence of B7, clonal anergy resulted (58).

Porcine B7-2 (PoB7-2) has been cloned from aortic endothelial cells (60). Following transient transfection of porcine B7-2, human umbilical vein endothelial cells strongly

costimulated IL-2 production by human T cells. This costimulation of human T cells by poB7-2 was shown to be as effective as costimulatory signals provided by human B7-1 or B7-2 and could be specifically blocked by huCTLA4Ig. Thus poB7-2 strongly contributes to the immunogenicity of porcine endothelium (60).

Although B7-1 and B7-2 mediated interactions appear to be central to the development of T cell specific immunity, additional costimulatory pathways of importance exist. The most crucial of which involves the CD40 and CD40 ligand (CD40L) interaction (34).

CD40 is a 50kDa surface glycoprotein belonging to the TNF-receptor superfamily. CD40 is expressed on various APC including among others, monocytes, dendritic cells and activated macrophages. Other cell types including endothelium also express CD40 (34). Its counter-receptor CD40L (CD154, gp39, TRAP) is a 33 kDa type II integral membrane protein (34,36) transiently expressed on activated CD4 T cells. The CD40-CD40L interaction has been demonstrated to play an important role in both the humoral and cellular arms of the immune response with a dominant role in B cell activation. Whilst cross linking of CD40 on B cells is essential for B cell growth and isotype switching, it also results in the upregulation of B7 expression (50). Levels of B7 expression (and thus APC capacity) of monocytes and dendritic cells are clearly unregulated following CD40 signalling (34). Data from CD40 knock-out mice demonstrated that CD40L signalling following ligation by CD40 plays an important role in T cell activation (61). Transfection of the murine P815 mastocytoma cells with CD40 (or B7-1) enabled previously non-stimulatory P815 cells to mediate the costimulation necessary for polyclonal T cell activation and the generation of cytokines (34). CD40-CD40L interactions have also been demonstrated to play a critical role in allograft rejection (62,63).

Resting B cells do not normally express B7-1/B7-2 at high levels until they are activated (50). Activation of B cells following simultaneous engagement of MHC-peptide/TCR and CD40-CD40L leads to the upregulation of B7 family members on B cells, thereby enhancing the stimulation and subsequent activation of T cells (34,36). Thus, the

CD40-CD40L interaction influences costimulatory activity by inducing expression of the B7 family of molecules and perhaps other costimulatory molecules, thereby playing a key role in T cell activation . The clear synergistic effects of CD40 and B7 indicate the importance of both costimulatory pathways for the initiation and amplification of T cell dependent immune responses (38). CD40-CD40L interactions have also been shown to play a crucial role in the generation of cytotoxic T lymphocyte (CTL) responses by modifying the functional status of a dendritic cell (64,65,66)

Extensive studies have demonstrated the importance of blocking B7-CD28 and/or CD40-CD40L interactions in the context of both allo and xenotransplantation. Data strongly supporting this includes the use of CTLA4Ig to block signalling via CD28-B7 resulting in enhanced graft survival and the prevention of chronic rejection in a rat cardiac allograft model (44,45) and a murine aortic allograft model (43). In these models, administration of CTLA4Ig caused partial (44) or complete (46) tolerance to graft antigen by inducing T cell anergy. Treatment of allo pancreatic islet transplants with anti-B7-2 and B7-1 antibody has also been demonstrated to inhibit transplant rejection (14). Similar results were obtained in models inhibiting CD40 signalling in a mouse cardiac allotransplant models (37,47,62). Two studies detailing the simultaneous blockade of signalling via CD28-B7 and CD40-CD40L prevented the onset of allorejection. Concurrent prolonged inhibition of both pathways completely abrogated the onset of chronic rejection in a mouse allo model (37) and in a skin and heart allo model (38).

In the realm of xenotransplantation, Lenshow and colleagues have, demonstrated long-term donor specific tolerance of human islets transplanted into mice with concomitant treatment with CTLA4Ig (46). Graft specific tolerance was demonstrated to be a direct consequence of inhibiting recognition via B7 expressing APC. In addition, Tran *et al* (67) deonstrated short term suppression with CTLA4-Fc treatment. There is limited data available on the simultaneous blockade of both pathways in the xenotransplantation context, with the prolonged survival of rat and porcine skin transplanted into murine recipients (63).

In vitro and *in vivo* data have clearly demonstrated that targeting the interactions mediated by either the CD28-B7 , CD40-CD40L, or both pathways has prevented the sensitisation of T cells to alloantigen and xenoantigen from engrafted tissue thereby prolonging graft survival ().

3.3 Peptide immunisation strategy

Previous *in vivo* studies using synthetic peptides conjugated to carrier molecules as immunogens have demonstrated the ability to generate the production of biologically active antibodies (68). There is now an extensive literature detailing peptide immunisation strategies which demonstrate enhancement of antibody production by carrier presentation(68-72). Thus, appropriate T cell epitopes can be used to prime T cells for subsequent help to B cells. Recent data has been published reporting the production of IgG by self-reactive B cells following immunisation with a self reacting antigen covalently coupled to a carrier molecule (70). Thereby demonstrating that B cell tolerance to self protein can be overcome.

As mentioned above, in order to be recognised by T cells, antigen (self or foreign) must be processed and presented by APC. B cells can act as highly potent APC following endocytosis of antigen via IgG receptors . In the presence of a full complement of activation signals (TCR engagement plus costimulation) T cell activation will occur resulting in the subsequent generation of antibody.

Peptides from self proteins are processed and presented to T cells in the same manner as foreign proteins, but because of T cell tolerance, presentation of self peptides does not normally result in T cell activation (70). The absence of T cell recognition may therefore explain, in part, why potentially reactive B cells fail to respond.

The ability to overcome B cell non-responsiveness to self peptides has recently been demonstrated by Dalum *et al* (69). An autoantibody response was generated by the

provision of additional T cell help in the form of a strong foreign carrier T cell epitope. Further studies have demonstrated that synthetic peptides conjugated to T cell carrier molecules are capable of overcoming B cell non-responsiveness if significant numbers of self-reactive B cells are present in the host (69,70). Insertion of a single foreign T cell epitope into the sequence of Ubiquitin, elicited strong autoantibody production directed against the native molecule (69). In an elegant study by Sad, using GnRH as a self protein chemically linked to diphtheria toxoid (DT) as the synthetic T cell epitope, autoantibodies were produced with specificity for native GnRH (71,72). Following the initial vaccination, the continued presence of the native GnRH *in vivo* maintained the production of Ab. Continued antibody production caused sterility in the immunised mice due to the sustained anti-GnRH antibody response maintained by the continued presence of the native molecule against which the specific B cells were producing antibody. The DT carrier provoked a helper T cell response to assist GnRH specific B cells and break B cell tolerance.

4. STATEMENTS OF INVENTION

The present invention involves the use of a foreign T cell carrier to exert significant influences on subsequent responses to molecules conjugated to the carrier. By such means autoantibody responses may be directed against costimulatory molecules in a xenotransplantation context.

According to the present invention there is provided a method of improving the tolerance of an animal, including a human being, to a xenograft, the animal having T cell mediated immunity, the method comprising causing the animal to raise an antibody against a xenomecuoe involved in the general of a rejection response in the animal, said antibody being raised by immunising the animal with a chimeric peptide comprising a T cell epitope against which the animal has immunity and a B cell epitope of said xenomecuoe.

Accordingly, xenograft specific tolerance is induced in transplant recipients by targeting the direct T cell mediated response by the use of chimeric peptide constructs to stimulate

the generation of specific anti-graft tolerance-promoting antibodies by the recipient prior to transplantation. By way of example, the chimeric peptides comprise a T cell epitope conjugated to sequences of porcine costimulatory molecules, B7-1, B7-2 and CD40. The presence of the engrafted tissue will then serve to maintain and perpetuate the production of antibody by the recipient's B cells.

The present invention also provide a chimeric peptide comprising a T cell epitope and a B cell epitope, said T cell being that of an animal, including a human being of a first species and said B cell being of an animal of a second species, said first and second species such that xeno transplantations suitable from an animal of said second species to an animal of said first species.

In addition, the present invention provides the use of a chimeric peptide improving the tolerance of an animal, including a human being, to a xenograph, the chimeric peptide being as defined above.

The potential benefits of the use of a chimeric peptide of the invention are that it avoids the need for injection of blocking antibodies or fusion proteins. Furthermore, the induction of a recipient antibody response circumvents the problems most commonly associated with administration of xenogeneic antibodies or fusions proteins, namely the immune response against the administered reagent.

5. SPECIFIC EMBODIMENTS

5.1 Cloning porcine costimulatory molecules

5.1.1 Cloning porcine B7-2

RNA was extracted from primary and transformed porcine cells using a standard protocol. mRNA was then reverse transcribed and porcine B7-2 (poB7-2) amplified from the cDNA by 35 cycles of PCR at 56^0 C with 1.5mM magnesium. The 5' and 3' primers GCATGGATCCATGGGACTGAGTAACATTCTCTTTG and GCATGTCGACTTAAAAATCTGTAGTACTGTTGTC respectively were designed on

the basis of the published poB7-2 sequence (60) to overlay the start and stop codons (Figure 2). A 956 base pair fragment was generated and subcloned into the BamH1 & Sal1 restriction sites of pbluescript. The nucleotide sequence was determined using standard m13 forward and reverse primers. The sequence of a single clone, CD86(i) is illustrated in Figure 3, with comparison to the published sequences from porcine (Figure 4), human and murine B7-2 (Figure 5). One base pair difference is detected between our clone, CD86(i), and the published sequence at the 3' prime end. This, however, is unlikely to be an important difference with respect to either poB7-2 expression or binding to its ligand. The predicted amino acid sequence of CD86(i) , compared to that of porcine, human and mouse B7-2 is shown in Figure 6.

5.1.2 Cloning porcine B7-1 and CD40

RNA extracted from phytohaemagglutinin (PHA) or poke-weed mitogen (PMW) stimulated porcine PBMC and transformed porcine endothelial cells is being used to amplify cDNA encoding the costimulatory molecules B7-1 and CD40. B7-1 Primers were designed on the basis of conserved areas following comparison of murine and human (29,49) sequences. External (lying outside the coding region) AGACCGTCTTCCTTTAG(3'i), TTGGATCCTCCATGTTATCCC (3'ii) and AGCATCTGAAGC (5') and internal (within the coding region) ATGGATCCTCCATTTTCCAACC (3') and TTGTCGACATCTACTGGC (5') primers have been designed as depicted in Figure 7. The generation of two 3' primers is due to significant differences between the human and murine sequences in the terminal coding regions. Resulting PCR fragments will be subcloned as described above using the restriction sites BamHI and SalI contained within the promoter sequence. Constructs will then be sent for sequence confirmation.

CD40 primers were designed in a similar manner following sequence alignment of published CD40 sequences from human, mice and cattle (73,74,75) as illustrated in Figures 8A & B. The 5' and 3' primer sequences are GGATCCTCACTGTCTCTCCTGCACTGAGATGCGACTCTCCTCTTTGCCGTCCG

TCCTCC and GAATTCATGGTTCTGTTGCCTCTGCAGTG respectively containing the BamHI and EcoRI restriction sites.

5.2 Generation of porcine costimulatory molecule expressing cell transfectants

The poB7-2 molecule (CD869(i)) has been subcloned into the eukaryotic expression vector pci.neo carrying the neomycin drug-selectable marker. This is being used to transfect M1 and M1.DR1 transformed murine cell lines using a standard calcium phosphate precipitation method. G418 resistant pci.neo expressing cells will be selected using dynabead purification and highly expressing clones will be selected by limiting dilution.

Stable poB7-2 M1 and P815 transfectants have been generated by this approach using the poB7-2 DNA construct supplied to us by Maher *et al* (Figure 9). transient transfections of M1 and P815 cells have been generated using our CD86(i) construct (Figure 10).

3 particular assays will be undertaken using the CD86(i) transfected cells.

(I) Comparative costimulatory function of poB7-2 with human B7-1 in the context of MHC restriction.

(II) Flow cytometric analysis of specific anti-poB7-2 antibodies in the sera of immunised mice.

(III) Generation of specific anti-poB7-2 monoclonal antibodies.

(I) Comparative *in vitro* analysis can be performed to determine the costimulatory function of poB7-2 or poB7-1 in the context of the human MHC class II molecule HLA-DR1, with that of human B7-1 or B7-2 in the context of DR1, in proliferation assays with human or porcine responders.

(II) Transfected P815 cells are crucial reagents for the detection of porcine anti-B7-2 antibody in the sera of immunised mice which have undergone the chimeric peptide immunisation regimen. Flow cytometric analysis with control or poB7-2 -transfected P815 cells, will reflect the specificity of sera for B7-2. Preliminary studies with C57BL-6

mice immunised with a pool of all nine B7-2 peptides have demonstrated the preferential binding of B7-2 peptide sera to porcine B7-2 transfected P815 cells (Figure 11).

(III) Mab with specificity for poB7-2 will be generated by immunisation of Balb/c mice with poB7-2 expressing P815 cells . The spleens from immunised mice will be fused with the NS0 fusion partner and successful fusion's selected by virtue of HAT selection. Flow cytometric staining of poB7-2 P815 transfectants with culture supernatants will enable the identification of MAb secreting cells. Cells will be grown in culture and the medium harvested for antibody purification by passage over Protein G following ammonium sulphate precipitation.

MAb with specificity for B7-1 and CD40 will be generated using the same protocol once the appropriate clones have been obtained. These MAb will provide valuable reagents for further characterising the expression of CS molecules on relevant porcine tissues.

5.3 Design and synthesis of poB7-2/OVA chimeric peptide constructs

Nine different peptides derived from the sequence of poB7-2 were initially selected for synthesis. Repeated batches of different peptides will be synthesised until successful molecules are obtained. Porcine B7-2 peptides, 6-22mer in size, were selected as determined by the predicted size of a B cell epitope. Peptides were selected for synthesis in combination with a T cell epitope OVA 323-339. B7-2 peptides were selected on the basis of 3D computer modelling (in collaboration with Paul Travers) and on the basis of predicted antigenicity and hydrophilicity using the SeqAid II computer software package. All of the nine peptides reflect linear epitopes. The positions of the nine peptides in the cloned poB7-2 sequence are indicated (Figure 12). Synthetic peptide sequences are detailed in Table 1

Table 1

Peptide Name	Peptide Sequence	Position
Peptide 1	ISQAVHAAHAEINEAGRSFDQATWTLR	81-90
Peptide 2	ISQAVHAAHAEINEAGRLPCHFTNSQ	32-40
Peptide 3	ISQAVHAAHAEINEAGRKGPHGLVPIHQMS	109-121
Peptide 4	ISQAVHAAHAEINEAGRGLVPIHQMS	113-121
Peptide 5	ISQAVHAAHAEINEAGRVQIKDKGSYQC	94-104
Peptide 6	ISQAVHAAHAEINEAGRCSSTQGYPEPQR	151-162
Peptide 8	ISQAVHAAHAEINEAGRKSQAYFNETGEL	21-32
Peptide 9	ISQAVHAAHAEINEAGRASLKSQAYFNET	17-29
Peptide 10	ISQAVHAAHAEINEAGRYMGRTSFDQATWT	76-88
Ova Peptide	ISQAVHAAHAEINEAGR	323-339

The peptide sequences and amino acid positions for peptides 1-10 relate to the position of the B7-2 peptide sequence within porcine B7-2. The amino acid position for the ova sequence is only indicated for the Ova peptide. A 17 amino acid peptide from chicken egg albumin (ovalbumin) was selected as the T cell epitope, OVA323-339 (ISQAVHAAHAEINEAGR). This epitope was selected on the basis of published reports for the generation of a H-2^b restricted T cell response (76,77).We have demonstrated the ability of C57BL-6 mice (H-2^b haplotype) to mount a proliferative response to both the native molecule and to the OVA 323-339 peptide following immunisation with whole ovalbumin (Figure 13). Peptides were generated on a peptide synthesiser (Genosys) and crude peptides were purified by HPLC to greater than 70% purity. Sera from OVA control immunised mice should ideally not recognise the 323-339 sequence, indicating that the T cell epitope is devoid of B cell determinants.

5.4 Tolerance induction

5.4.1 *In vivo* tolerance induction strategy

C57BL-6 mice will be immunised with whole ovalbumin in CFA, followed by either control peptide (OVA peptide) or test peptides (OVA-B7-2 constructs) for three weekly immunisations. Blood will be collected following sacrifice and sera prepared using a

(iii) Following the successful cloning of all three costimulatory molecules, a combined strategy will be employed to block all three CS molecules by immunisation with appropriate peptides. It is predicted that blocking all three CS molecules will be sufficient to inhibit T cell mediated destruction of the graft by the direct pathway resulting in prolongation of islet graft survival. The tolerance induction strategy detailed in this application is directed against the direct xenorecognition pathway. Thus , if islet survival is to be significantly enhanced above that of controls, other additional strategies may be necessary to target the indirect pathway.

The results obtained with B7-2 to date, demonstrate the ability of synthetic B7-2 peptides conjugated to a known T cell helper epitope to generate the production of anti-pocine B7-2 antibody *in vivo*. These antibodies if directed towards the binding site between B7 isoforms and CD28, in association with antibodies directed against CD40-CD40L will block the costimulation of human T cells with direct anti-pig xenoreactivity thereby prolonging islet survival in a xenotransplantation context.

Having established the suitability of such an approach in a pig islet to mouse *in vivo* model, studies would progress to pig to primate transplantation systems prior to clinical trials.

5.5 Adaptations for clinical use of these strategies

For clinical applicability the following requirements will be necessary:

(I) selection of a suitable T cell epitope to replace OVA. One candidate molecule is tetanus toxiod (TT) which is a widely used antigen for use in human immunisation strategies (68,86). The prior immunisations of most adults with TT is an additional benefit to this strategy as memory T cells are already present in the circulation.

(ii) An efficient and rapid screening method will be required to detect the presence of anti-donor (pig) B7-2 antibodies in the absence of a specific B7-2 directed T cell response generated by the recipient which would accelerate graft rejection.

6. SUMMARY OF SPECIFIC EMBODIMENTS

The above examples relate to a novel strategy to inhibit costimulation by porcine cells of human T cells with direct anti-pig xenoreactivity. This is of particular importance in the context of xenotransplantation of porcine organs due to the expression of costimulatory molecules on porcine endothelial, as well as bone marrow-derived antigen presenting cells.

Recipients will be immunised with hybrid synthetic peptides comprising a T cell epitope conjugated to sequences of the porcine costimulatory molecules, CD80, CD86 and CD40. Peptides will be selected that induce antibodies specific for regions of the costimulatory molecules involved in binding to their counter-receptors on human cells (CD28 and CD154), and therefore capable of blocking the delivery of costimulation. Once the antibody response has been induced, the transplanted organ will recall this response due to the expression of the costimulatory molecules, thereby sustaining this response, and providing an endogenous mechanism of costimulatory blockade.

standard technique. Presence of specific mouse anti-porcine B7-2 IgG and/or IgM Ab will be detected by one of two strategies.

Peptide ELISAs will be used to screen for the presence of anti-peptide antibody in the sera. Peptides are coated to plates by virtue of aldehyde linkages to allow free access of Ab to the peptide (78). Plates will be coated with individual peptides or the ova control peptide to enable the identification of specific peptides of interest. To detect reactivity of sera with the native B7-2 molecule expressed on the surface of PoB7-2 transfected P815 cells, flow cytometry will be performed following surface staining. Having identified a candidate peptide of interest (peptide ELISA positive and recognising native B7-2) the sera will be used to try to inhibit *in vitro* T cell proliferative responses. This will determine whether the antibody is a blocking antibody. *In vivo* studies will then be performed using the islet transplant system. Antibodies which recognise the native molecule but fail to block a proliferative response will still be useful polyclonal antibody reagents.

To date, initial immunisations involved two groups of mice, one received a pool of all nine B7-2 peptides, and one receiving ova control peptide. The harvested sera were screened by peptide ELISA (Figure 14) which enabled the identification of potential peptides of interest. Peptides 2, 4 and 6 clearly demonstrate preferential binding to B7 peptide than to ova control. The sera has also demonstrated enhanced binding to poB7-2 transfected cells (Figure 11). Peptide 4 was selected as a candidate peptide and used in a subsequent immunisation protocol. Immunisation with peptide 4 alone clearly produced a significant level of IgG with specificity for peptide 4 in the sera of immunised mice (Figure 15). The specificity of the sera for peptide 4 and not to ova control is demonstrated in Figure 16. The ability of sera from peptide 4 immunised mice to specifically recognise the native porcine B7-2 molecule expressed on the surface of porcine B7-2 transfected P815 cells is illustrated in Figure 17. Untransfected control P815 cells do not stain with the Peptide 4 sera, neither do control or transfected cells incubated with ova peptide sera. Similar protocols will be followed with peptides 2 and

6. These data clearly demonstrate the ability of this technique to generate anti-peptide antibody directed against an amino acid sequence, by virtue of a carrier T cell epitope. An identical strategy will be followed with peptides designed on the basis of porcine CD40 and porcine B7-1 once the DNA sequence encoding these molecules has been elucidated.

5.4.2 Functional assessment; prolongation of pancreatic islet xenograft survival

Islet xenografts being non-vascular are rejected solely by T cell mediated mechanisms (79,80), thereby providing an ideal system to study modulation of T cell mediated reactions. A very clear role for cell mediated rejection of islets has been demonstrated and is reported to be greater than the comparable alloresponse (80). Transplantation of porcine pancreatic islets to mice is an established procedure, which is well documented in the literature (80-83). Preliminary studies within this laboratory have demonstrated a decrease in hyperglycaemia (Figure 18) following transplantation of pancreatic islets from large white pigs under the kidney capsule of C57BL-6 mice rendered diabetic by intraperitoneal administration of streptozotocin. Further optimisation of the isolation procedure (84,85) is required to enable purification of fully functional islets. Transplanted islets usually survive between 6-10 days in the absence of any immunosupression. Successful modulation of direct T cell mediated xenorejection will be monitored by prolongation of islet survival beyond day 10, with comparison to the appropriate controls.

Plans for investigation include:

(i)Survival and functional assessment of transplanted islets from control and test mice. Survival of islets ie: graft tolerance, will be determined by reversion to and maintenance of normoglycaemia as monitored by using a Reflolux S blood glucose meter. Prior to islet transplantation mice will follow the tolerance induction strategy detailed above.

(ii) To determine whether our tolerance induction strategy has induced graft-specific T cell tolerance, identical or third party islets can be transplanted under the contra-lateral kidney capsule.

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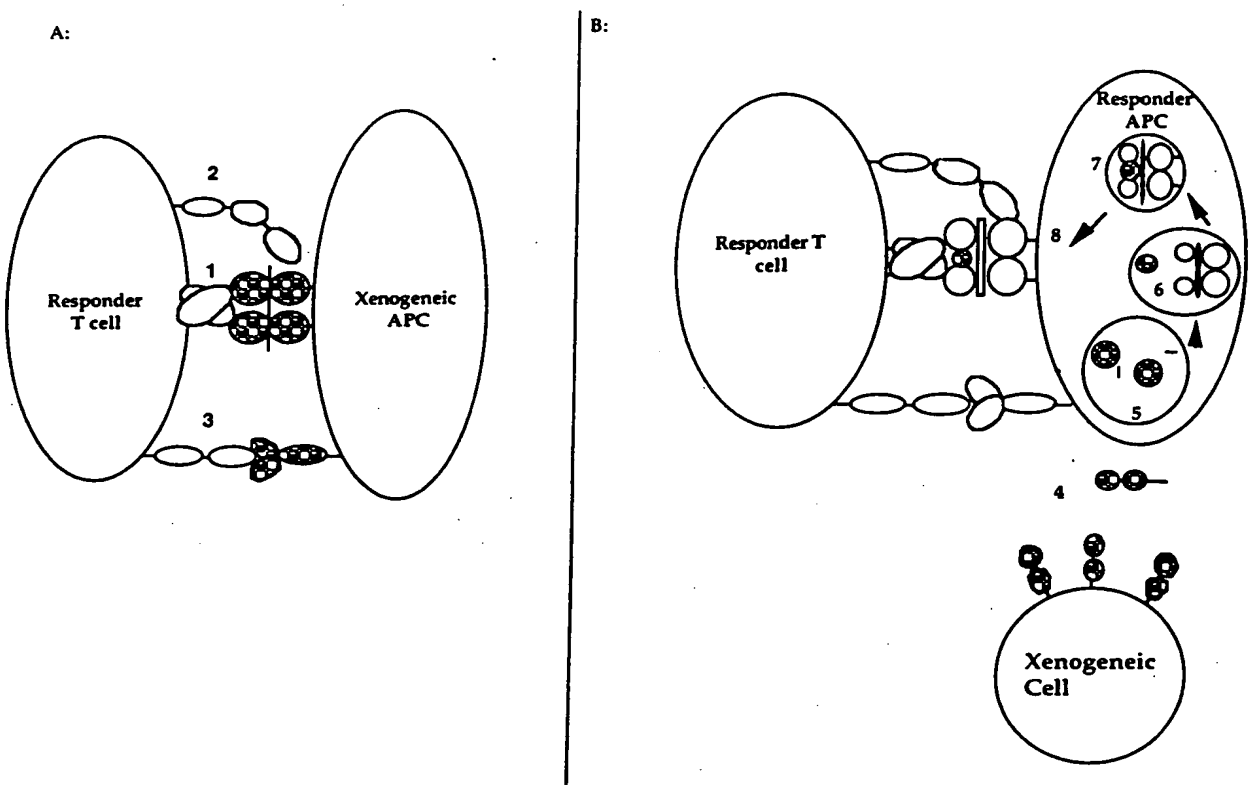
5

Figure 1



A: Diagrammatic representation of direct xenorecognition.

The types of molecular interactions necessary for efficient direct xenorecognition are numbered 1 - 3.

1 Cognate interaction between TCR on responder T cell and MHC molecules on xenogeneic antigen presenting cells.

2 Non cognate interaction between co-receptors CD4 and membrane proximal domains of MHC class II, and CD8 and α3 domains of MHC class I.

3 Non cognate interactions between accessory and costimulatory molecules. Important interactions are between B7 family (APC) and CD28 (T), LFA-3 (APC) and CD2 (T), and ICAM-1 (APC) and LFA-1 (T)

B; Diagrammatic representation of indirect xenorecognition

Xenoantigens (4), released by xenogeneic cells, are taken up and processed (5) into peptide fragments by specialised antigen presenting cells (6) before binding to MHC class II molecules (7) and display on the cell surface (8) for presentation to xenospecific self-class II MHC-restricted T cells.

Figure 1: Diagramatic comparison of direct and indirect xenorecognition pathways.

GCATGGATCCATGGGACTGAGTAACATTCTCTTTG

1 **ATG**GGACTGAGTAACATTCTCTTTGTGATGGTCCTCCT

39 GCTCTCTGGTGCTGCCTCCTTGAAAAGTCAGGCATATTTCAATGAGA

86 CTGGAGAACTGCCGTGCCATTTTACAAACTCGCAGAACCTAAGCCTG

133 GATGAGCTGGTCATATTTTGGCAGGACCAGGATAACCTGGTTCTCTA

181 CGAGCTATACCGAGGCCAAGAGAAGCCTCATAATGTTAATTCCAAG

227 TATATGGGTCGCACAAGCTTTGACCAGGCCACCTGGACCCTGAGACT

274 CCACAACGTTCAAATCAAGGACAAGGGCTCATATCAATGTTTCATC

321 CATCATAAAGGGCCGCATGGACTTGTTCCTATCCACCAGATGAGTTC

368 TGACCTATCATTGCTTGCTAACTTCAGTCAACCTGAAATAAACCTAC

415 TTACTAATCACACAGAAAATTCTGTCATAAATTTGACCTGCTCATCT

462 ACACAAGGCTACCCAGAACCCCAGAGGATGTATATGTTGCTAAATA

509 CGAAGAATTCAACCACTGAGCATGATGCTGACATGAAGAAATCTCA

556 AAATAACATCACGGAACTCTACAATGTATCAATCAGGGTGTCTCTT

602 CCCATCCCTCCCGAGACAAATGTGAGCATCGTCTGTGTCCTGCAACTT

649 GAGCCAAGCAAGACACTGCTTTTCTCCCTACCTTGTAATATAGATGC

696 AAAGCCACCTGTGCAACCCCCTGTCCCAGACCACATCCTCTGGATTGC

743 AGCTCTACTTGTAACAGTGGTCGTTGTGTGTGGGATGGTGTCCTTTGT

790 AACACTAAGGAAAAGGAAGAAGAAGCAGCCTGGCCCCTCTAATGA

837 ATGTGGTGAAACCATCAAAATGAACAGGAAGGCGAGTGAACAAAC

884 TAAGAACAGAGCAGAAGTCCATGAACGATCTGATGATGCCCAGTGT

931 GATGTTAATATTTTAAAGACAGCCTCAGAT**GACAACAGTACTACAG**

GACAACAGTACTACAG

978 ATTTT**TAA**TTAAAGAGTAAACTCC

ATTTTTAAGTCGACATGC

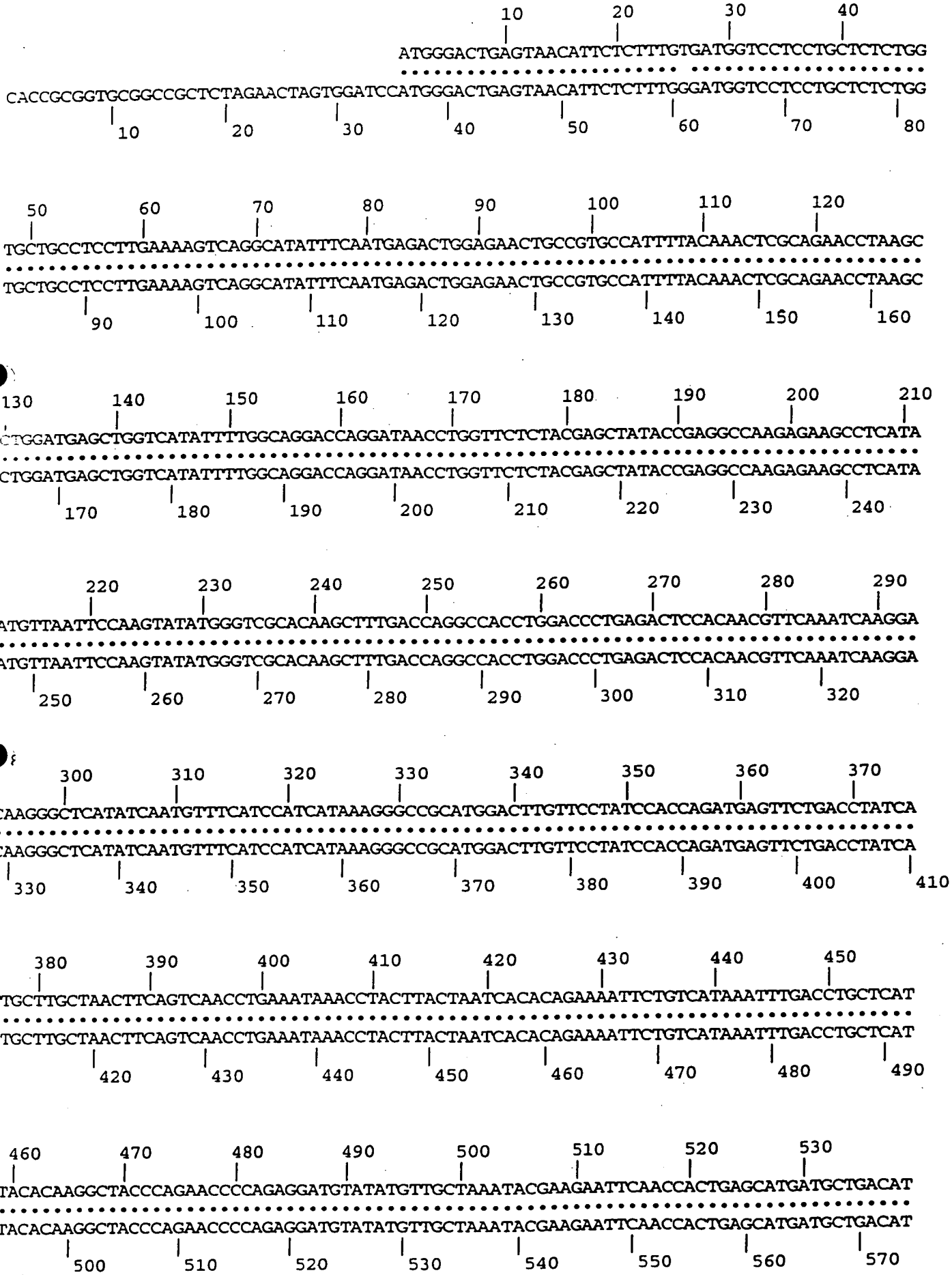
Figure 2: Position of 5' and 3' primers (highlighted in bold type)with respect to the published coding sequence of porcine CD86. The underlined sequences ATG and TAA represent the start and stop codons respectively.

```
   1  CACCGCGGTG CGGCCGCTCT AGAACTAGTG GATCCATGGG ACTGAGTAAC
  51  ATTCTCTTTG GGATGGTCCT CCTGCTCTCT GGTGCTGCCT CCTTGAAAAG
 101  TCAGGCATAT TTCAATGAGA CTGGAGAACT GCCGTGCCAT TTTACAAACT
 151  CGCAGAACCT AAGCCTGGAT GAGCTGGTCA TATTTTGGCA GGACCAGGAT
 201  AACCTGGTTC TCTACGAGCT ATACCGAGGC CAAGAGAAGC CTCATAATGT
 251  TAATTCCAAG TATATGGGTC GCACAAGCTT TGACCAGGCC ACCTGGACCC
 301  TGAGACTCCA CAACGTTCAA ATCAAGGACA AGGGCTCATA TCAATGTTTC
 351  ATCCATCATA AAGGGCCGCA TGGACTTGTT CCTATCCACC AGATGAGTTC
 401  TGACCTATCA GTGCTTGCTA ACTTCAGTCA ACCTGAAATA AACCTACTTA
 451  CTAATCACAC AGAAAATTCT GTCATAAATT TGACCTGCTC ATCTACACAA
 501  GGCTACCCAG AACCCCAGAG GATGTATATG TTGCTAAATA CGAAGAATTC
 551  AACCACTGAG CATGATGCTG ACATGAAGAA ATCTCAAAAT AACATCACGG
 601  AACTCTACAA TGTATCAATC AGGGTGTCTC TTCCCATCCC TCCCGAGACA
 651  AATGTGAGCA TCGTCTGTGT CCTGCAACTT GAGCCAAGCA AGACACTGCT
 701  TTTCTCCCTA CCTTGTAATA TAGATGCAAA GCCACCTGTG CAACCCCCTG
 751  TCCCAGACCA CATCCTCTGG ATTGCAGCTC TACTTGTAAC AGTGGTCGTT
 801  GTGTGTGGGA TGGTGTCCTT TGTAACACTA AGGAAAAGGA AGAAGAAGCA
 851  GCCTGGCCCC TCTAATGAAT GTGGTGAAAC CATCAAAATG AACAGGAAGG
 901  CGAGTGAACA AACTAAGAAC AGAGCAGAAG TCCATGAACG ATCTGATGAT
 951  GCCCAGTGTG ATGTTAATAT TTTAAAGACA GCCTCAGATG ACAACAGTAC
1001  TACAGATTTT TAAGTCGACC TCGAGGGGGG GCCCGGTACC AGCTTTTGTT
```

Figure 3: Nucleotide sequence of CD86(i) obtained by RT-PCR amplification of cDNA extracted from a transformed porcine endothelial cell line A8.

Figure 4: Comparison of the nucleotide sequence of CD86(i) with the published sequence for porcine CD86.

Figure 4: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10 20 30 40 50 60 70 80
50 60 70 80 90 100 110 120
TGCTGCCTCCTTGAAAAGTCAGGCATATTTCAATGAGACTGGAGAACTGCCGTGCCATTTTACAAACTCGCAGAACCTAAGC
TGCTGCCTCCTTGAAAAGTCAGGCATATTTCAATGAGACTGGAGAACTGCCGTGCCATTTTACAAACTCGCAGAACCTAAGC
90 100 110 120 130 140 150 160
130 140 150 160 170 180 190 200 210
CTGGATGAGCTGGTCATATTTTGGCAGGACCAGGATAACCTGGTTCTCTACGAGCTATACCGAGGCCAAGAGAAGCCTCATA
CTGGATGAGCTGGTCATATTTTGGCAGGACCAGGATAACCTGGTTCTCTACGAGCTATACCGAGGCCAAGAGAAGCCTCATA
170 180 190 200 210 220 230 240
220 230 240 250 260 270 280 290
ATGTTAATTCCAAGTATATGGGTCGCACAAGCTTTGACCAGGCCACCTGGACCCTGAGACTCCACAACGTTCAAATCAAGGA
ATGTTAATTCCAAGTATATGGGTCGCACAAGCTTTGACCAGGCCACCTGGACCCTGAGACTCCACAACGTTCAAATCAAGGA
250 260 270 280 290 300 310 320
300 310 320 330 340 350 360 370
CAAGGGCTCATATCAATGTTTCATCCATCATAAAGGGCCGCATGGACTTGTTCCTATCCACCAGATGAGTTCTGACCTATCA
CAAGGGCTCATATCAATGTTTCATCCATCATAAAGGGCCGCATGGACTTGTTCCTATCCACCAGATGAGTTCTGACCTATCA
330 340 350 360 370 380 390 400 410
380 390 400 410 420 430 440 450
TTGCTTGCTAACTTCAGTCAACCTGAAATAAACCTACTTACTAATCACACAGAAAATTCTGTCATAAATTTGACCTGCTCAT
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420 430 440 450 460 470 480 490
460 470 480 490 500 510 520 530
CTACACAAGGCTACCCAGAACCCCAGAGGATGTATATGTTGCTAAATACGAAGAATTCAACCACTGAGCATGATGCTGACAT
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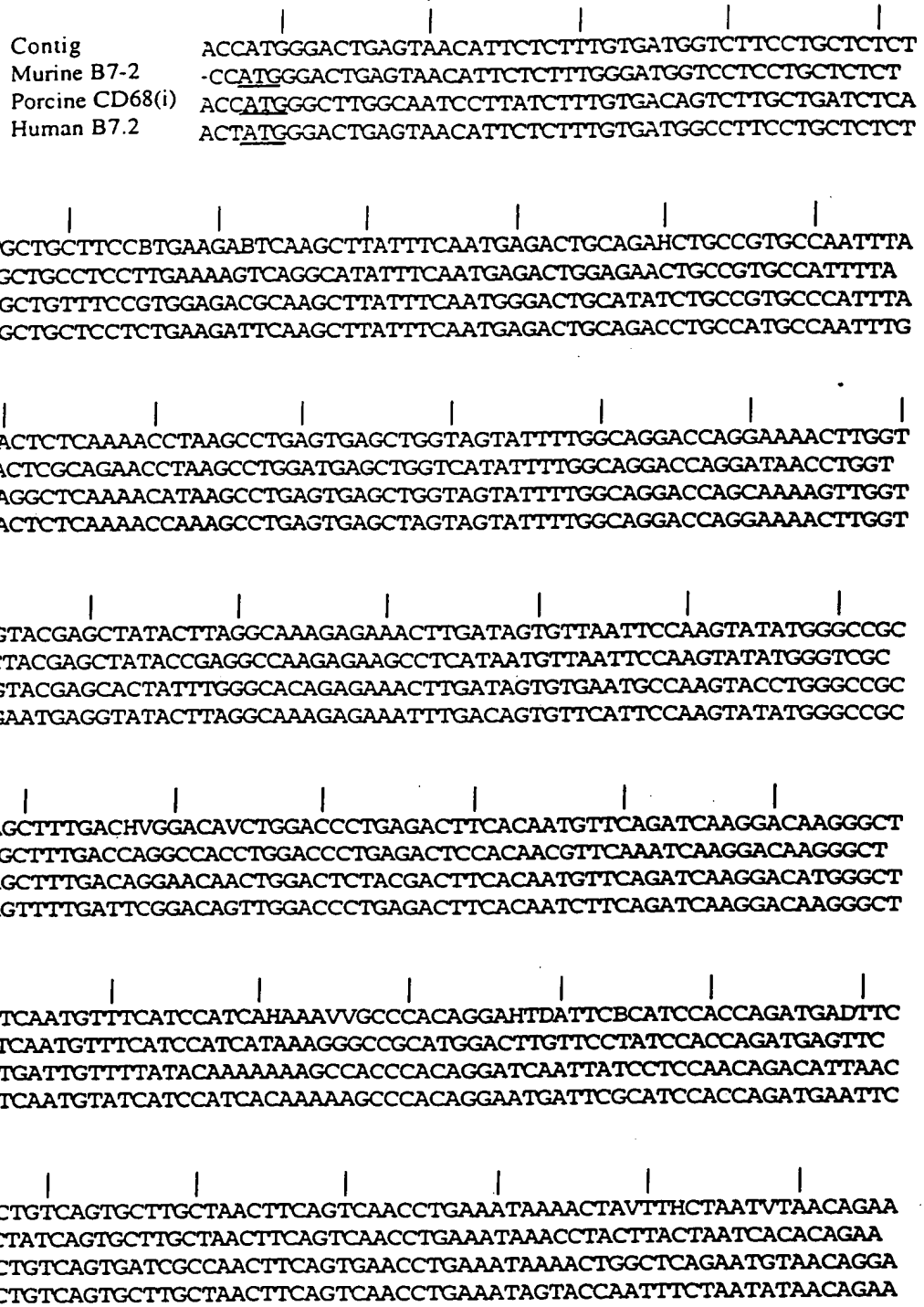
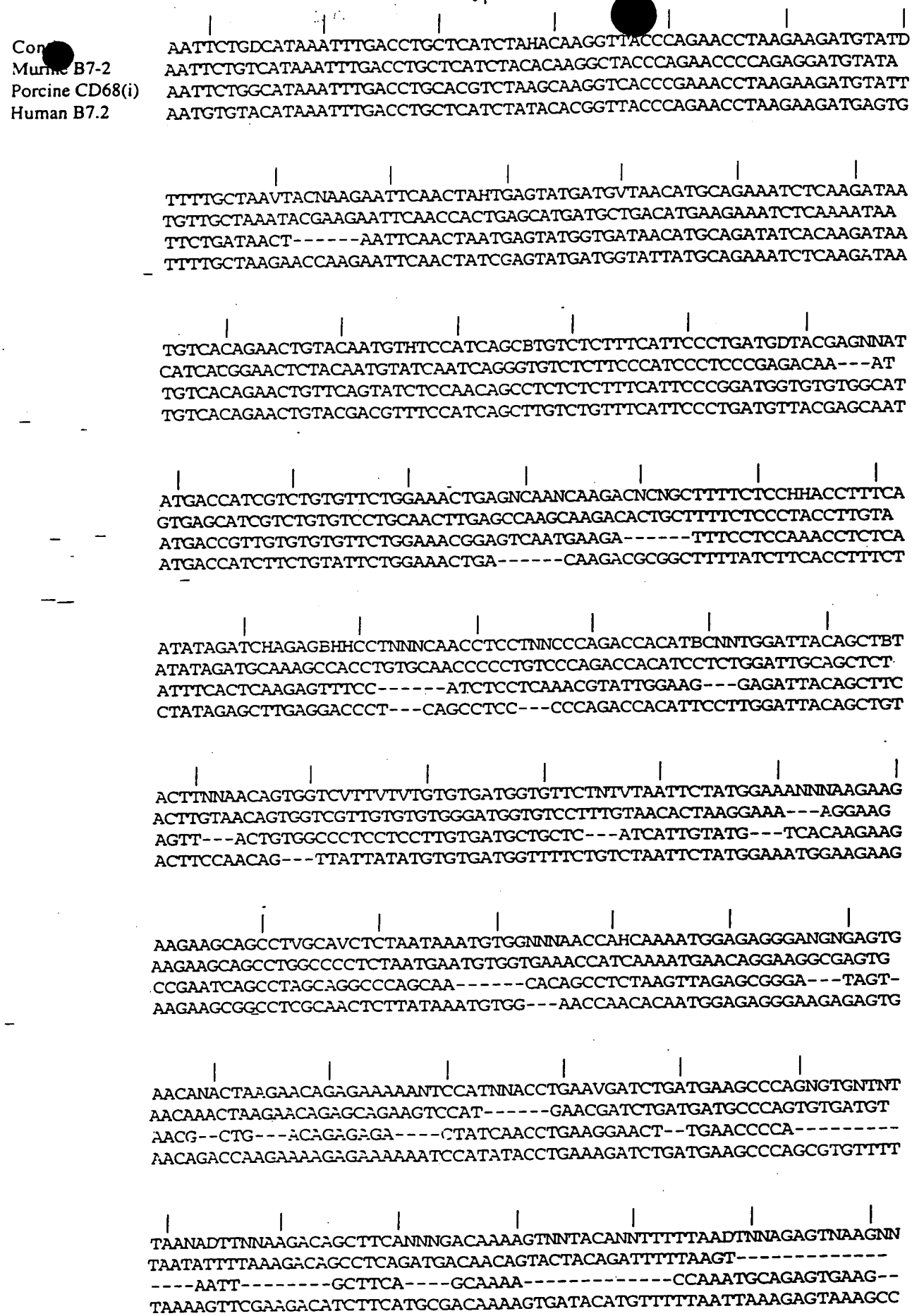


Figure 5: Comparison of CD86(i) with published sequences for murine and human CD86. Sequence continues overleaf.


Con
Murine B7-2
Porcine CD68(i)
Human B7.2
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AATTCTGTCATAAATTTGACCTGCTCATCTACACAAGGCTACCCAGAACCCCAGAGGATGTATA
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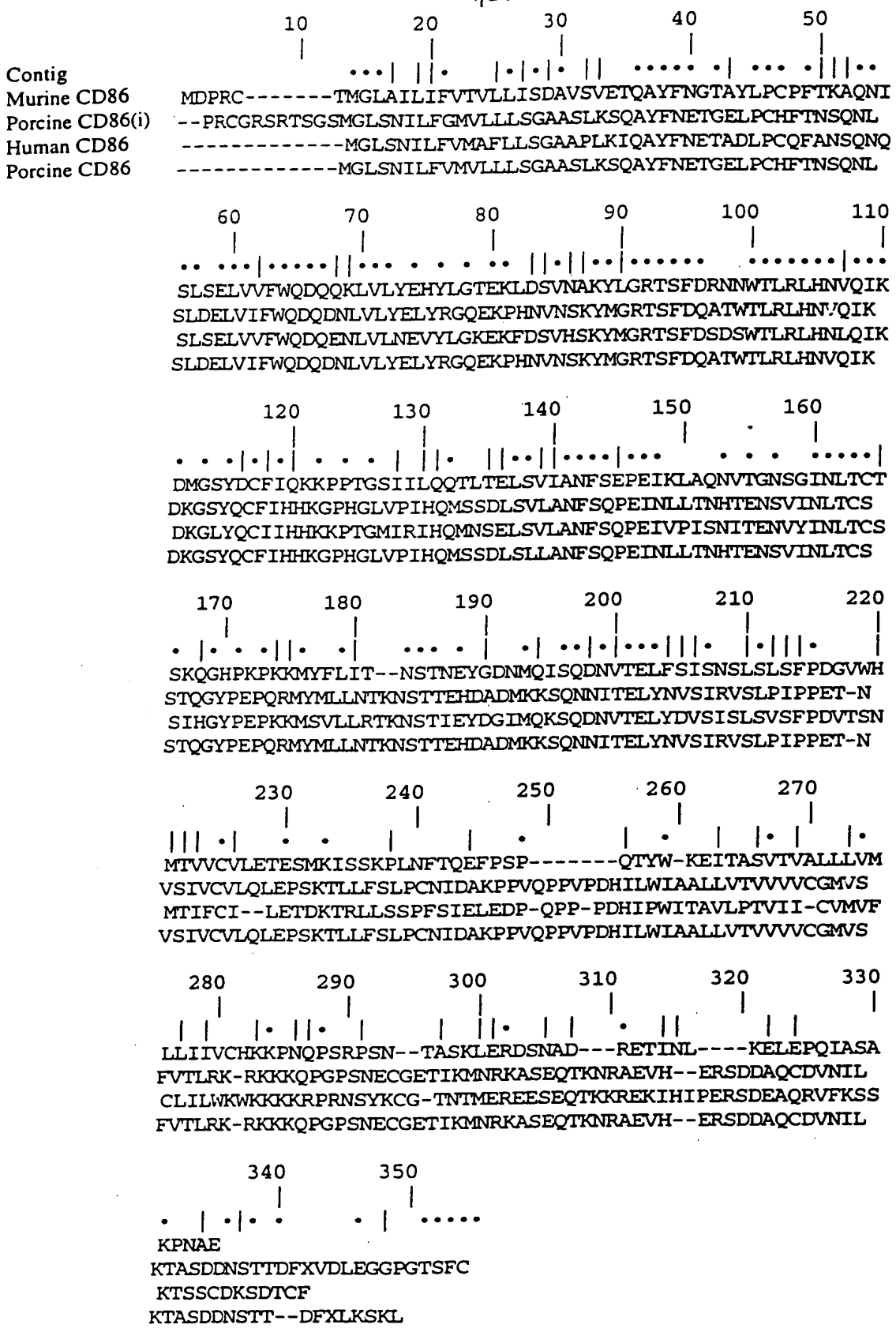
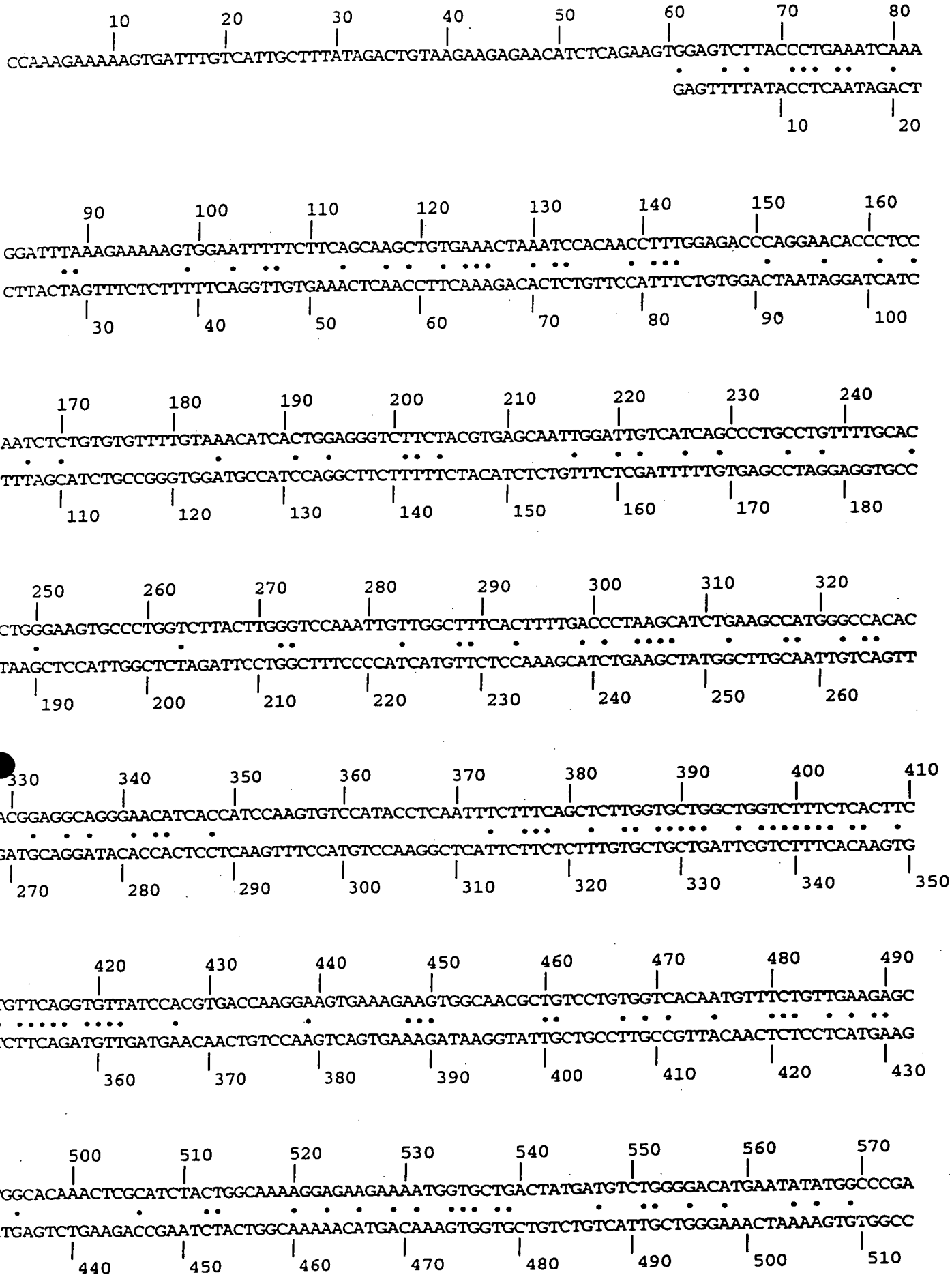


Figure 6: Predicted amino acid sequence for CD86(i) compared with those for pig, human and mice.

Figure 7: Position of 5' and 3' internal and external porcine B7-1 primers with respect to human and murine B7-1 nucleotide sequences. Primer sequences are underlined and labelled as follows. Internal primers (A) and external primers (B).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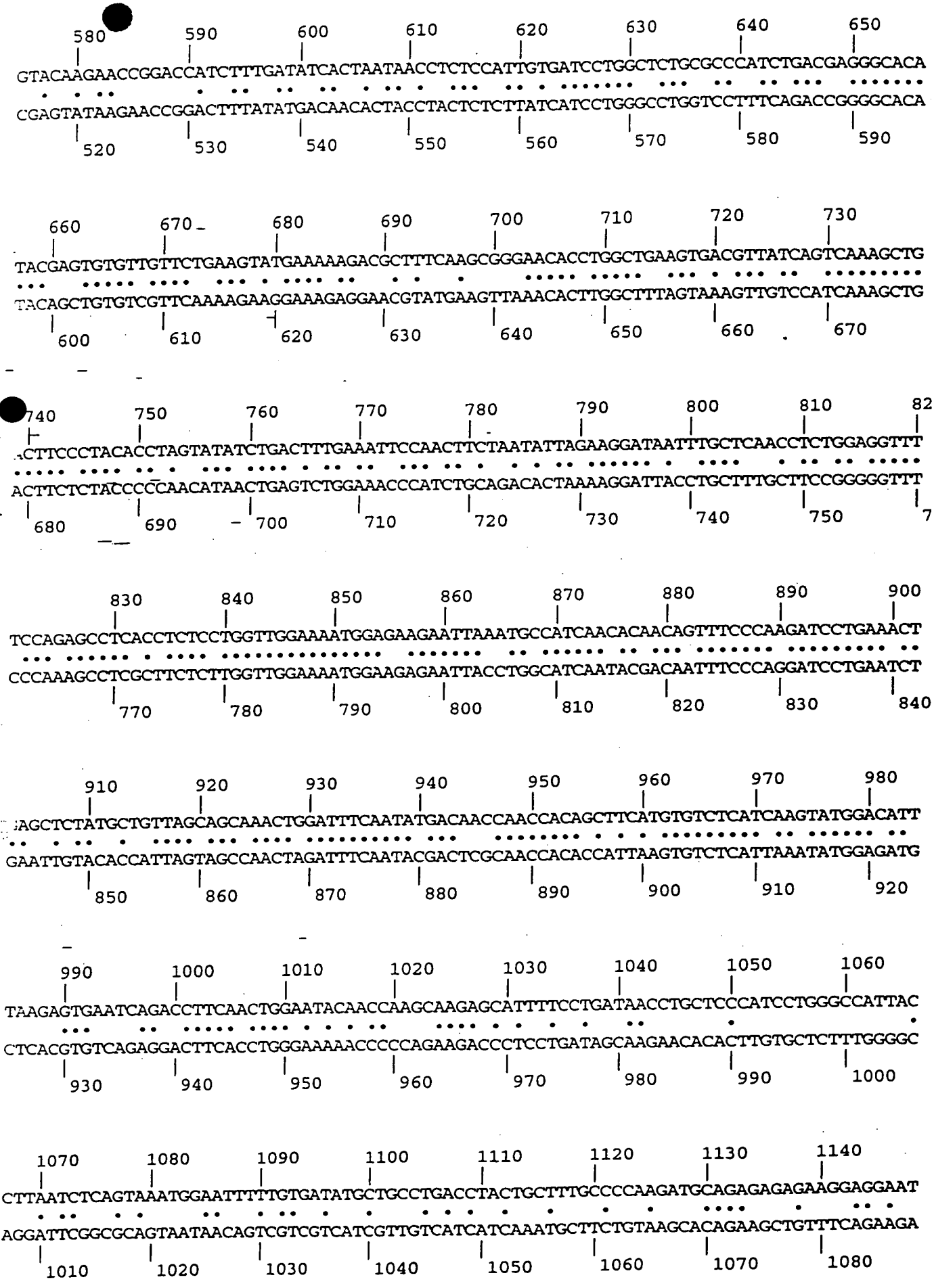
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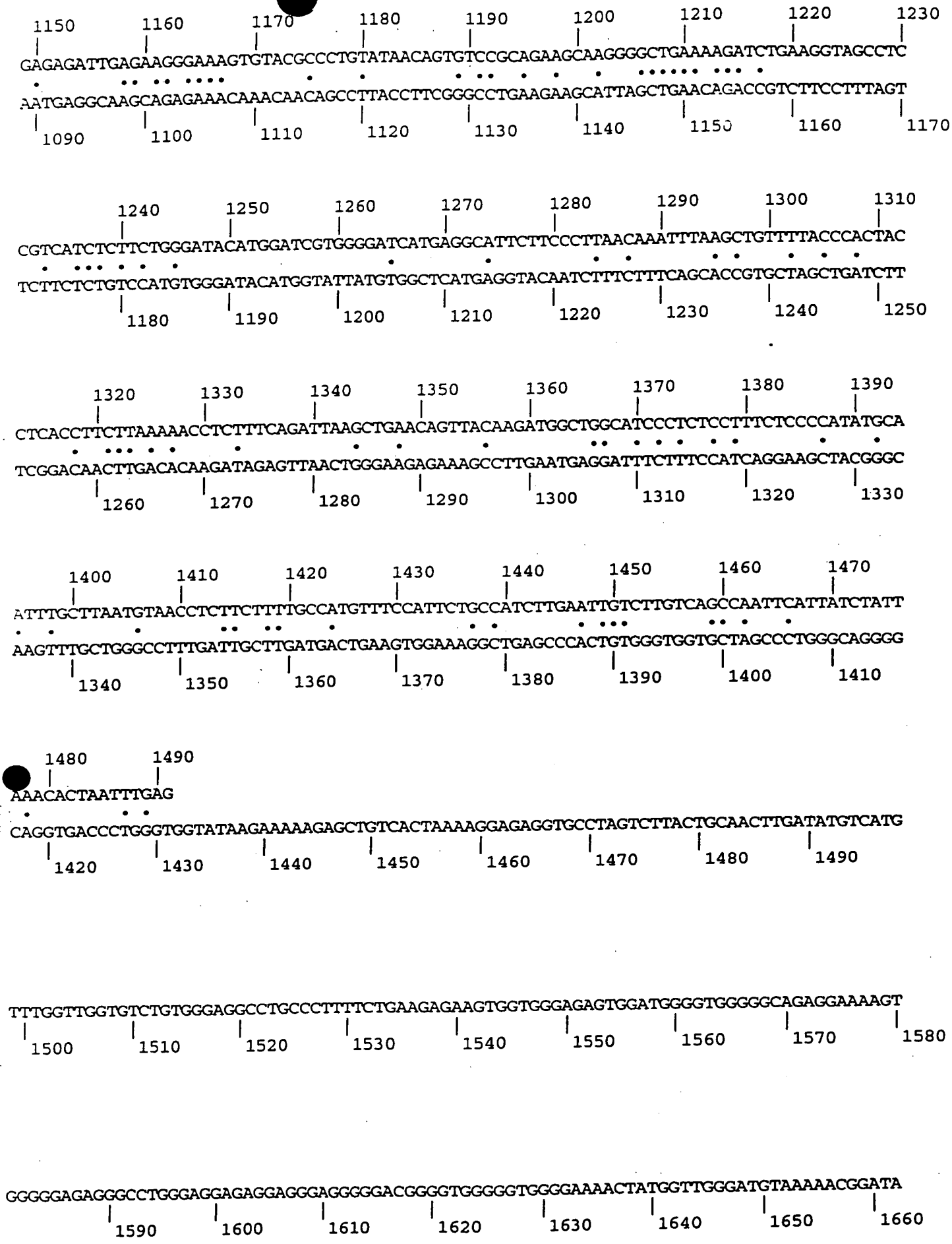
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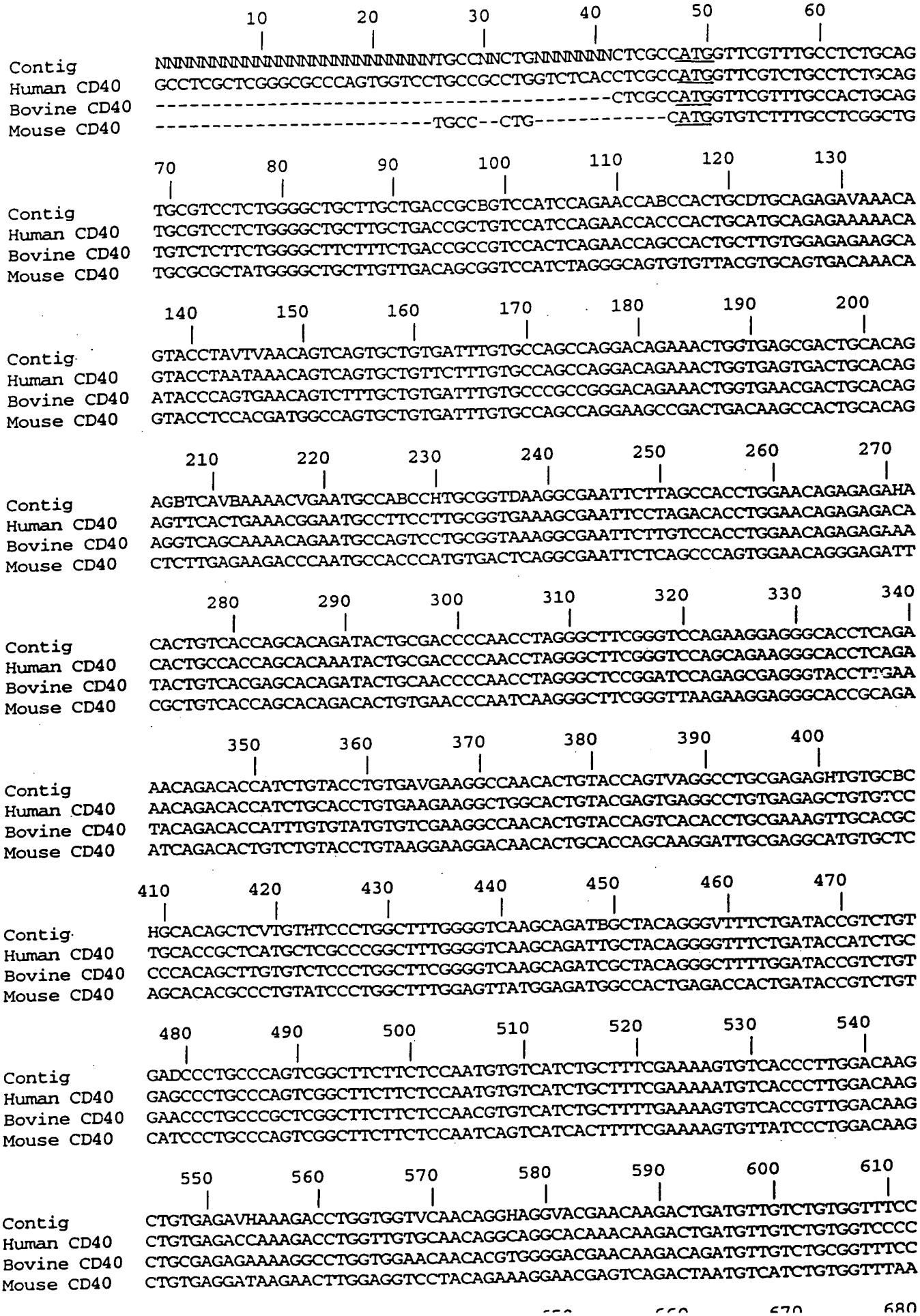
Figure 8A: CD40 nucleotide sequence comparison between human, murine and cattle sequences.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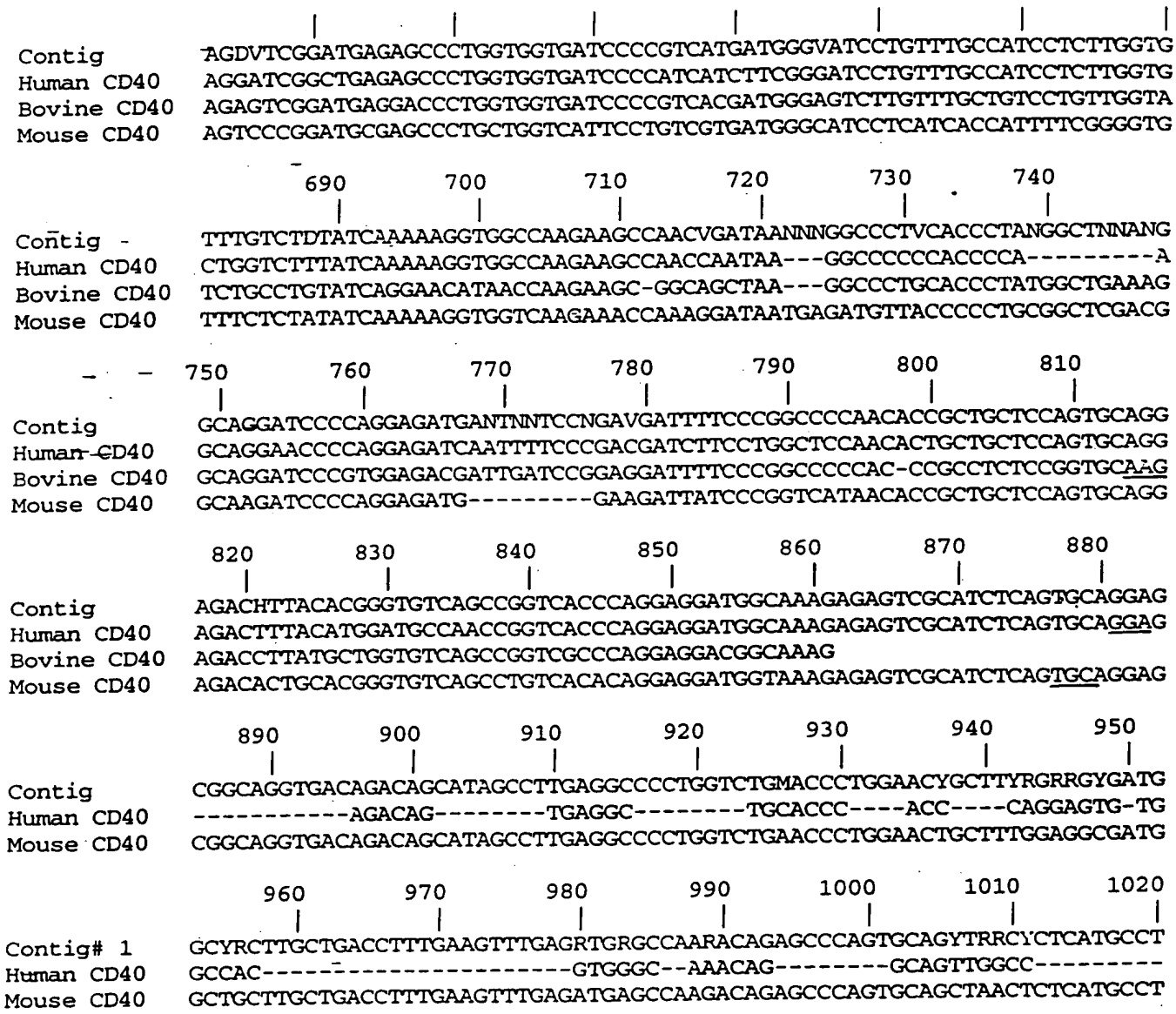
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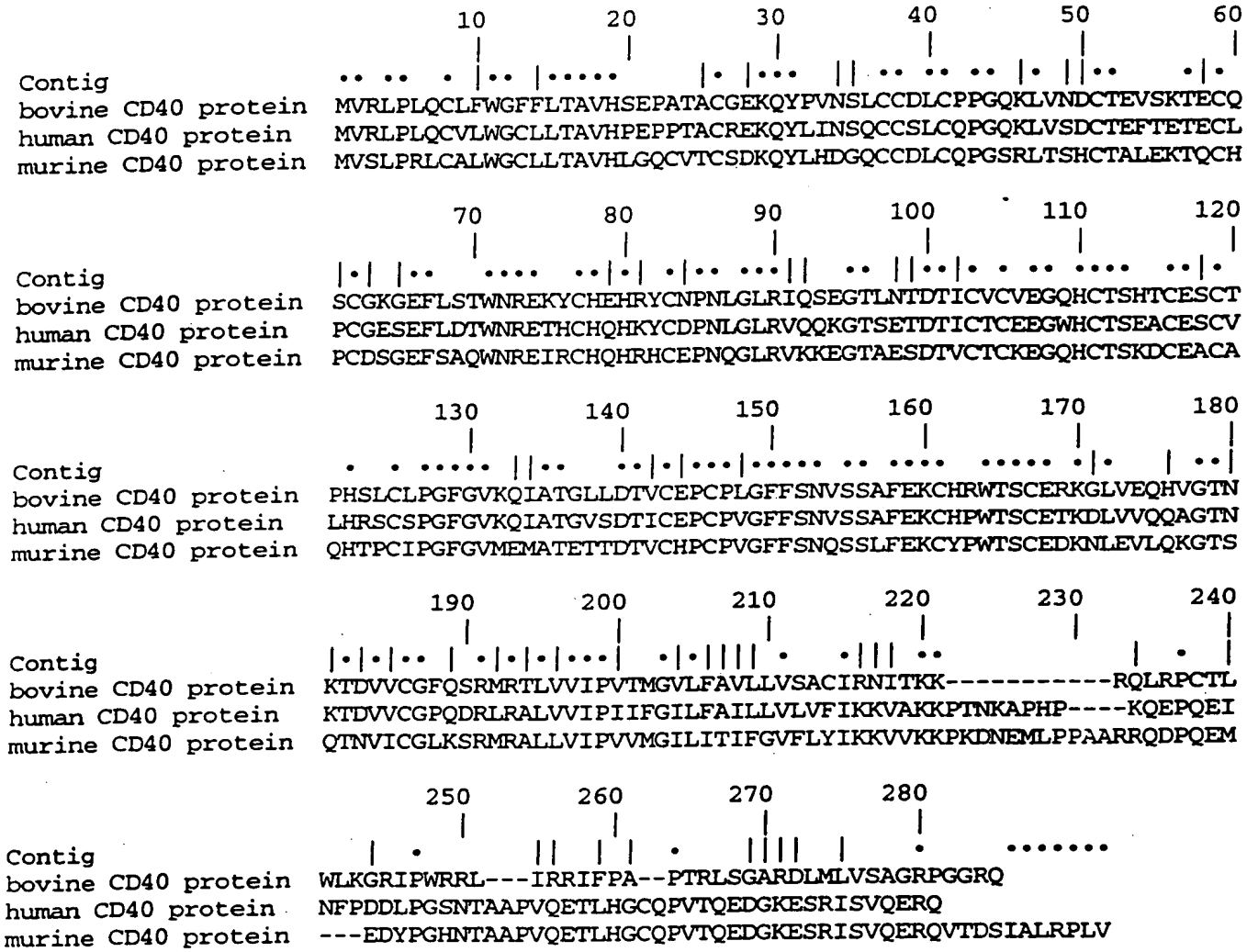
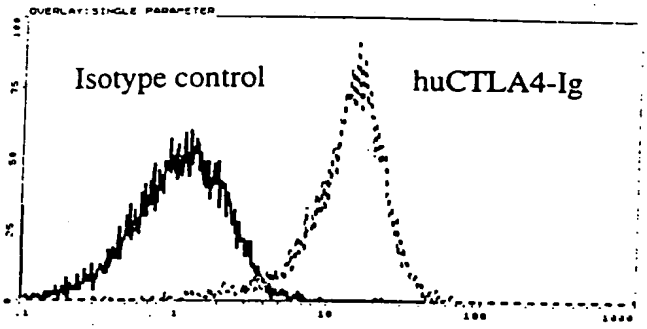


Figure 8B: Amino acid comparison between human, murine and cattle CD40 sequences.

A

Non-transfected control cells

Transfected cells



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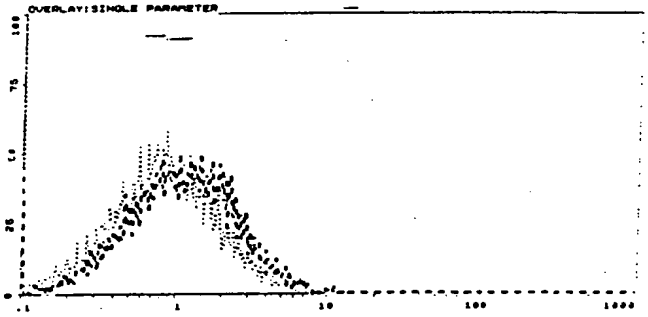

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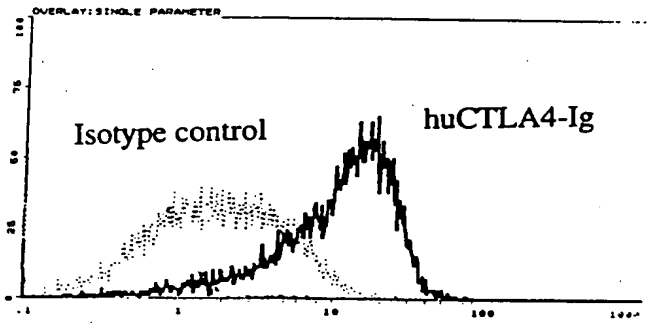
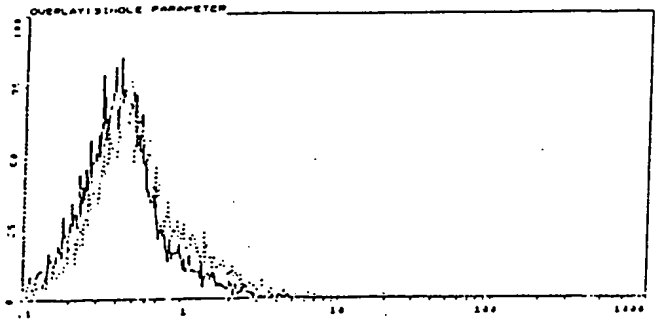
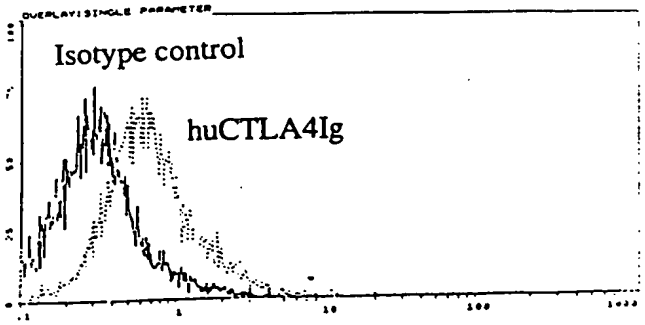


Figure 9: M1-poB7-2 (A) and P815-poB7-2 (B) clones generated by calcium phosphate transfection followed by dynabead selection and cloning by limiting dilution. Expression of B7-2 on the surface of transfected or control cells as determined by fluorescence activated cell sorting.2.5 x 10^5 cells were stained with Mab to B7-2 (huCTLA4Ig) or isotype control (huIg) at 1 g/ml. After washing, cells were incubated with goat anti-mouse Ig-FITC conjugate, fixed with 1% paraformaldehyde and analysed on a Coulter counter.

Non-transfected control cells

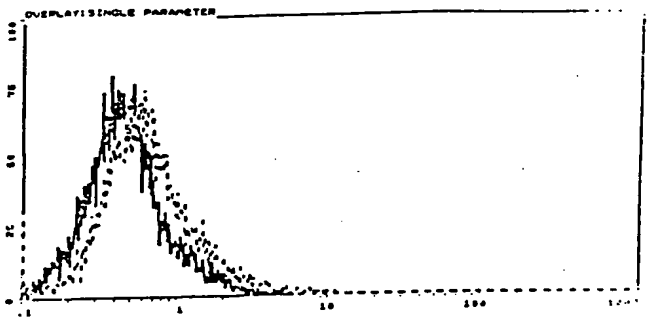
Transfected cells





Non-transfected control cells

Transfected cells



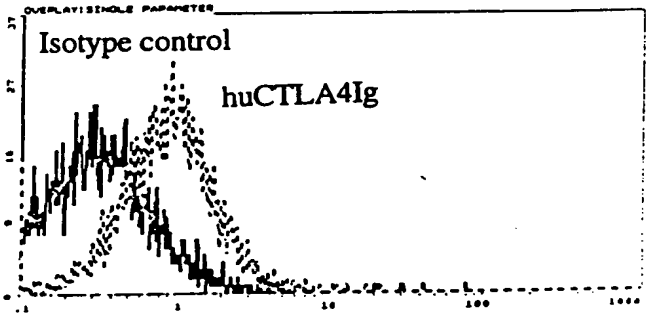
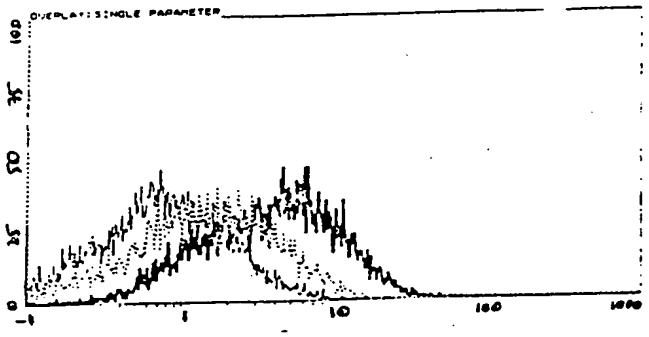


Figure 10: Transient transfections of M1 (A) and P815 (B) cells with CD86(i) by calcium phosphate precipitation .Surface expression of B7-2 on transfected or control cells was determined by fluorescence activated cell sorting.48 hours after transfection, 2.5 x 10^5 cells were stained with Mab to B7-2 (huCTLA4Ig) or isotype control (huIg) at 1 g/ml. After washing, cells were incubated with goat anti-mouse Ig-FITC conjugate, fixed with 1% paraformaldehyde and analysed on a Coulter counter..

A



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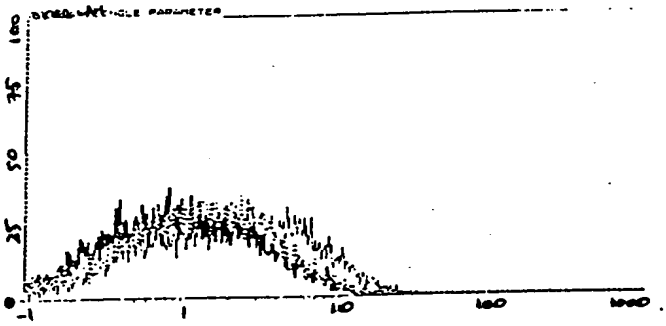


Figure 11: Flow cytometric analysis of porcine B7-2 transfected P815 cells following staining with porcine B7-2-specific sera or ovalbumin peptide control sera. 2.5 x 10^5 P815 cells were stained with 1/100 of each sera from B7-2 peptide (A) or ova control peptide (B) immunised mice. After washing, cells were incubated with goat anti-mouse IgG (H & L)-HRP and subsequently, Streptavidin-FITC. Cells were fixed with 1% paraformaldehyde and analysed on a Coulter counter.

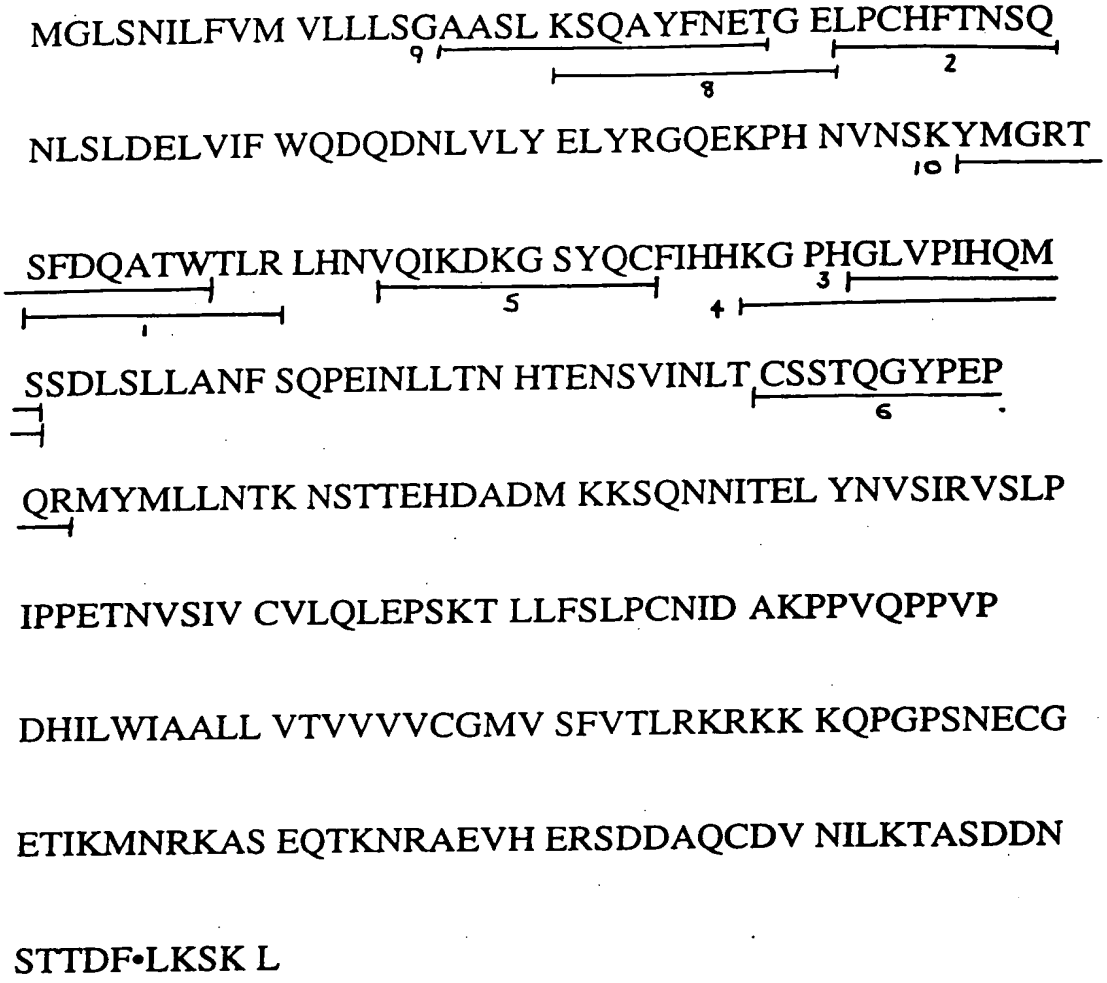
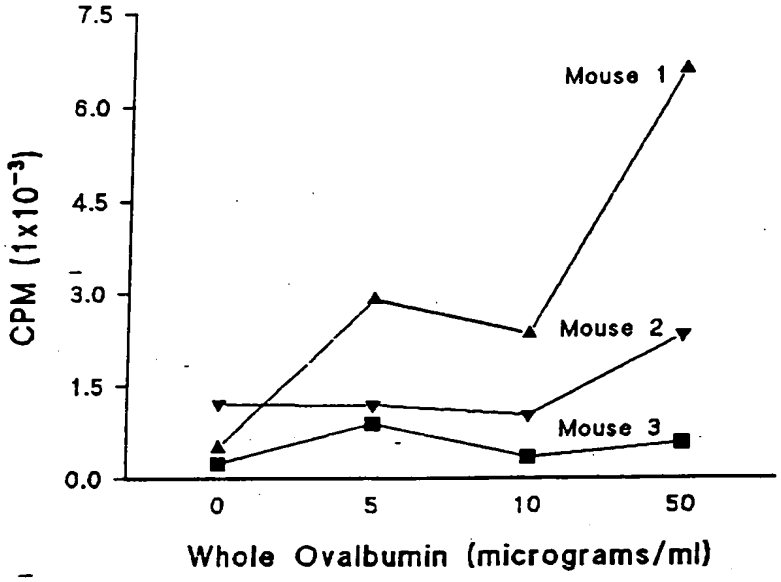


Figure 12: Positions of the nine B7-2 peptides with respect to the predicted amino acid sequence of porcine B7-2

A



B

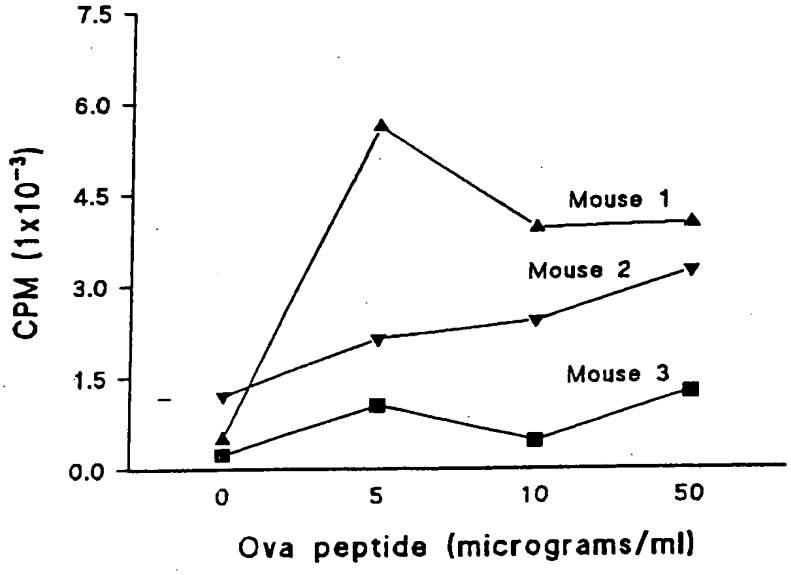
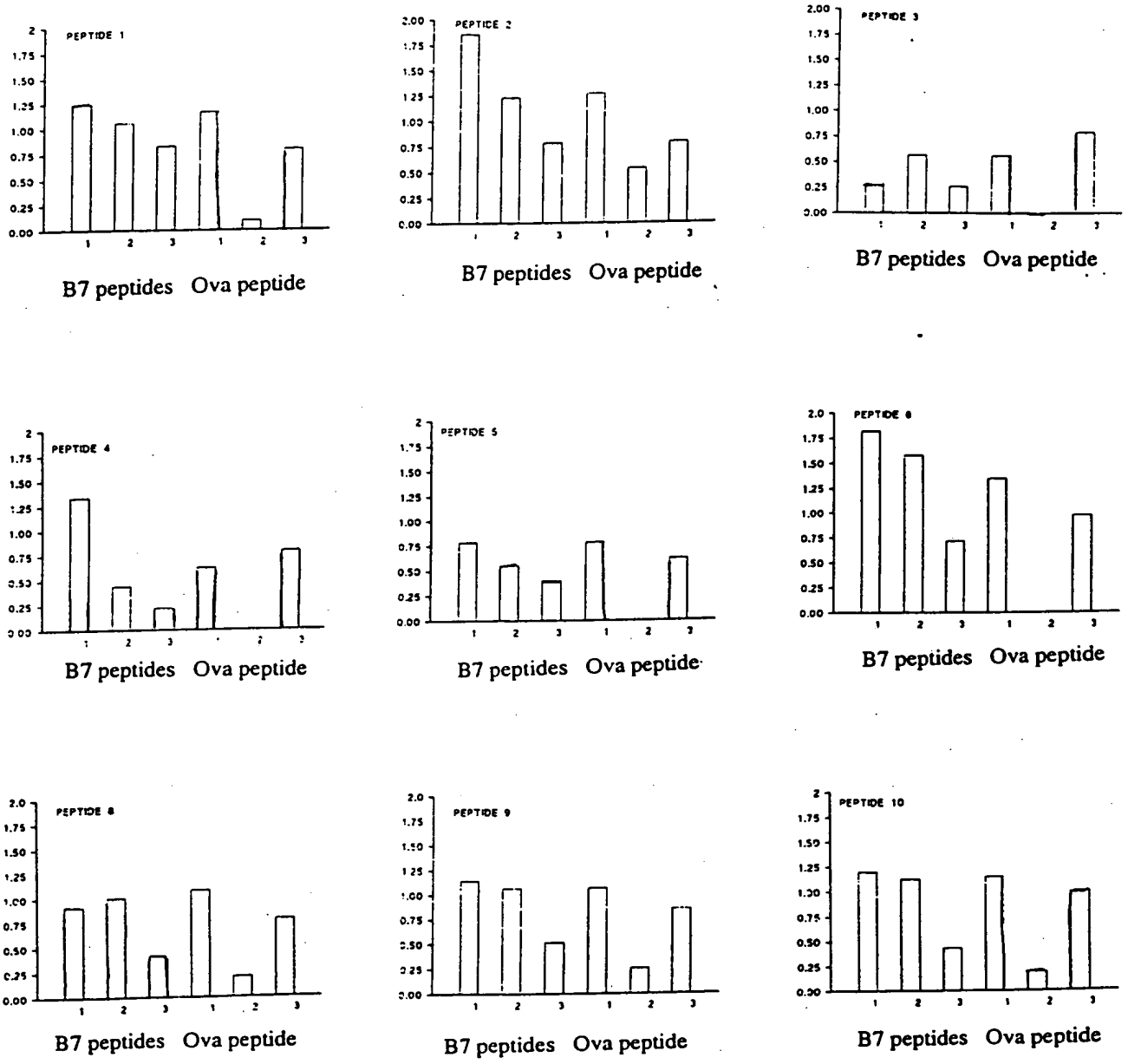


Figure 13: Comparison of *in vitro* T cell proliferation response to whole ovalbumin (A) or $Ova_{323-339}$ peptide (B). 2.5 x 10^5 T cells and 2.5 x 10^5 APC were plated per well with the indicated concentrations of whole ovalbumin or ova peptide. Cells were cultured for 72 hours in a total volume of 200 l 10% RPMI. T cell proliferation was assayed by the incorporation of ^{3}H-thymidine.



1:300 dilution of sera from immunised mice

Figure 14: Differential binding of B7-2 specific peptide sera or ova control sera as determined by Peptide ELISA. 96 well plates were pre-coated with the nine individual B7-2 specific peptides (P1-6 ; P8-10). Sera harvested from 3 individual B7-2 peptide (Bars 1-3), or 3 individual Ova control peptide (Bars 4-6) immunised mice were then screened for binding. Sera were detected by subsequent incubations with goat anti-mouse IgG-Biotin, Streptavidin-HRP and then developed with TMB. Plates were read at 450nm. Values have been adjusted for binding to no-peptide control plate and represent means for duplicate wells.

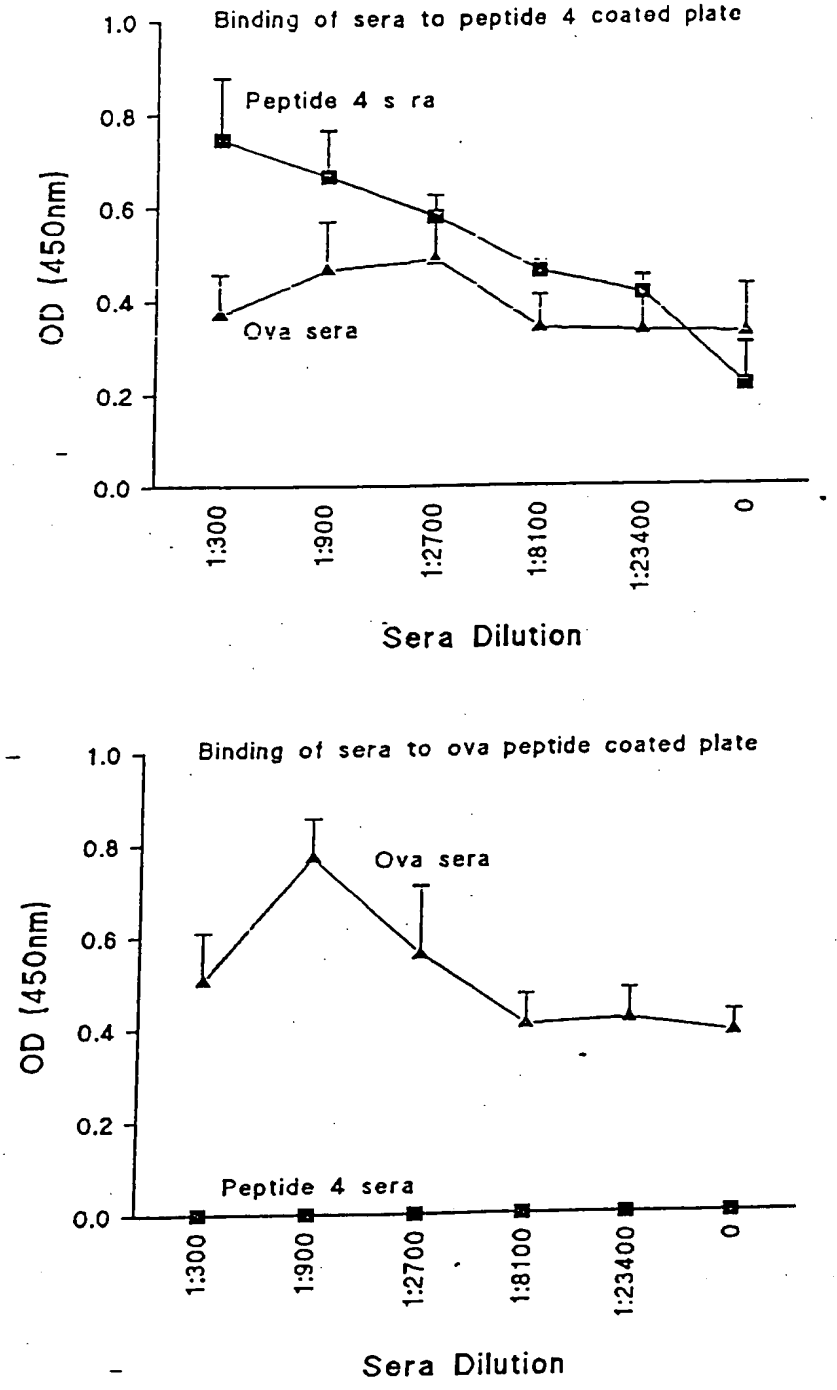
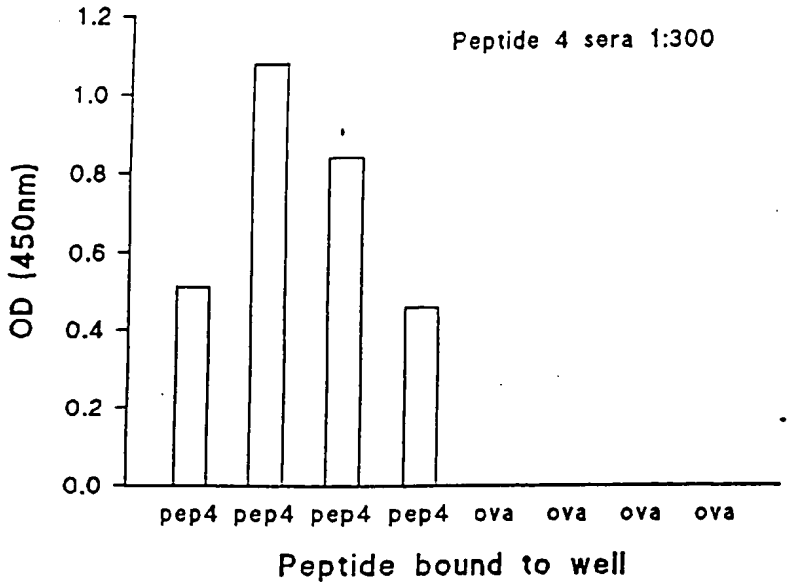


Figure 15: Differential binding of B7-2 specific sera or ova control sera as determined by Peptide ELISA. 96 well plates were pre-coated with either the B7-2 specific peptide Pep4. Ova control peptide (OVA) or no peptide. Sera harvested from peptide 4, or Ova peptide immunised mice were then screened for binding. Sera were detected by subsequent incubations with goat anti-mouse IgG-Biotin, Streptavidin-HRP and then developed with TMB. Plates were read at 450nm.: Values represent means +/- SEM for 4 mice per group, in duplicate wells. Values have been adjusted for binding to no-peptide control plate.Sera were measured over a range of dilutions.



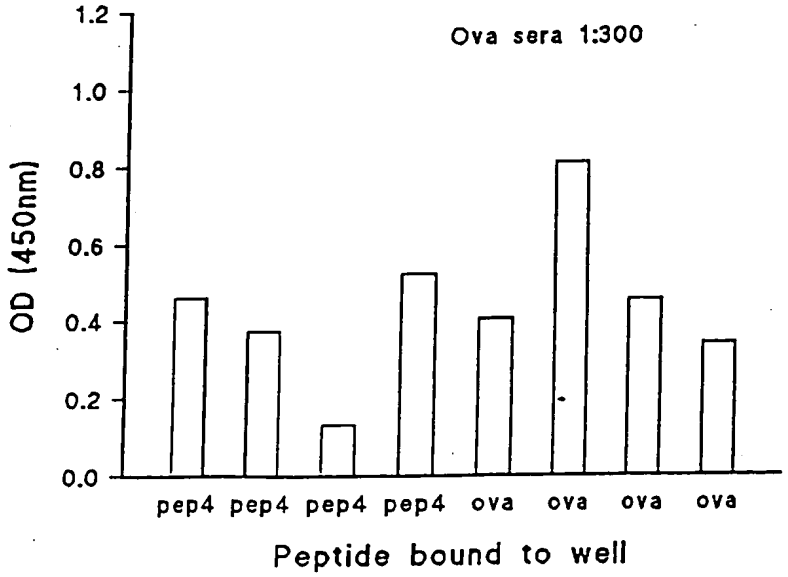


Figure 16 : Differential binding of B7-2 specific sera or ova control sera as determined by Peptide ELISA. 96 well plates were pre-coated with either the B7-2 specific peptide Pep4, Ova control peptide (OVA) or no peptide. Sera harvested from peptide 4, or Ova peptide immunised mice were then screened for binding. Sera were detected by subsequent incubations with goat anti-mouse IgG-Biotin, Streptavidin-HRP and then developed with TMB. Plates were read at 450nm. Values have been adjusted for binding to no-peptide control plates and represent means for duplicate wells for individual mice at 1:300 dilution of the sera.

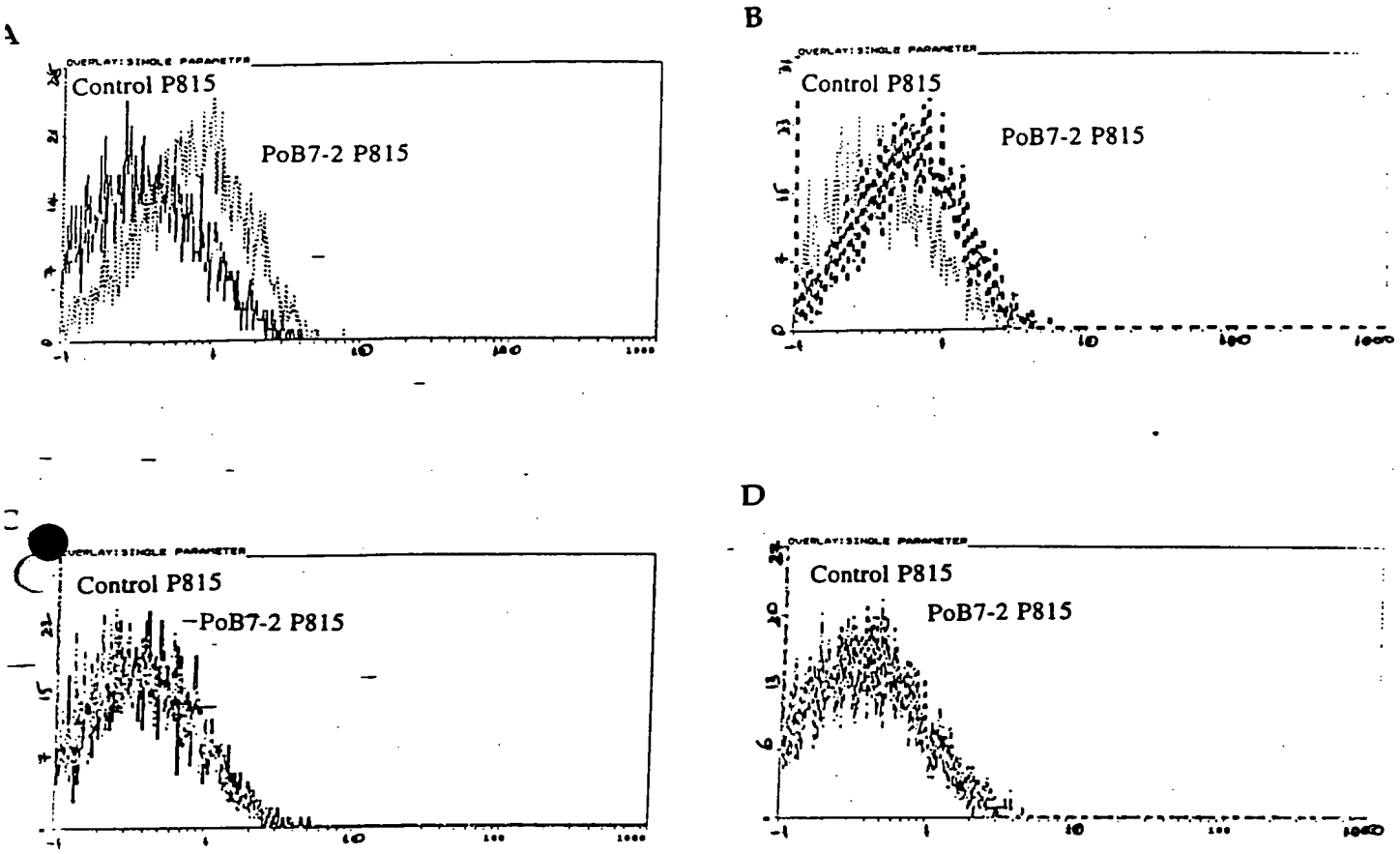


Figure 17: Flow cytometric analysis of porcine B7-2 transfected, or control untransfected P815 cells following staining with sera from peptide 4 or ovalbumin peptide control sera. 2.5 x 10^5 P815 cells were stained with 1µl of sera from 4 different mice immunised with either B7-2 peptide 4 (Figures A & B) or ova control peptide sera (Figures D & E). After washing, cells were incubated with goat anti-mouse IgG (H & L)-HRP and subsequently, Streptavidin-FITC. Cells were fixed with 1% paraformaldehyde and analysed on a Coulter counter.